SOMATOSTATIN-EXPRESSING NEURONS IN THE BED NUCLEUS OF THE STRIA TERMINALIS

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Tiivistelmä/Referat – Abstract

The bed nucleus of the stria terminalis (BNST) is currently widely studied due to its impact in the anxiety-, stress-, and fear-related behaviours, as well as in addiction. The BNST is highly heterogeneous brain area constituting of set of subnuclei and a variety of neuron populations, properties of which have only partially been revealed by the earlier research. One of the neuron populations, on which only a very little research has been conducted, is the somatostatin (Sst) expressing neurons, highly abundant in the anterodorsal part of the BNST (adBNST), especially in oval and juxtacapsular nuclei of the BNST. This work aims to elucidate the connectivity of this Sst-neuron population, and their role in the behaviours related to BNST activation, particularly the anxiety-, reward-, and drug withdrawal-related behaviours.

To specifically study the somatostatin neuron population in the adBNST, I targeted the neurons using stereotaxic delivery of AAV-vectors encoding a myristylated green fluorescent protein (GFP) for neuronal tracing to Sst-Cre-tdTomato reporter line mice (n=2), and Cre-inducible hM3Dq-DREADDs to Sst-IRES-Cre mice (n=21), with Cre-inducible mCherry fluorescent protein as a control (n=20). The mice were treated with activation-inducing 1.0 mg/kg i.p. clozapine-N-oxide (CNO) 30 min prior to the behavioural tests. To assess acute anxiety-like behaviour, I used the elevated-plus maze paradigm and a modified open field test, in which a novel object is introduced to the arena in the middle of the trial. To study the potential effect on reward-associated behaviours, I used the biased conditioned place preference (CPP) test, and for the withdrawal-linked behaviours, we used a method to precipitate the withdrawal symptoms with naltrexone in subchronically morphine-treated mice (n=9 hM3Dq, n=8 control).

The neuronal tracing revealed that the adBNST Sst-neurons project to areas known to partake in stress and fear reactions as well as in autonomic and homeostatic control. Namely, projections were seen in medial and central amygdaloidal nuclei, lateral hypothalamus, periaqueductal grey, ventral pallidum, and parabrachial nucleus. In the elevated-plus maze, the CNO-induced activation of the Sst-neurons did not have any effect on the locomotor activity of the mice between the groups. At the same time, Sst activation did not seem to have any significant effect on the time the mice spent in the open arms, nor in the exploratory activities, like the frequency of the head dips or the stretch-attend postures. In line with these results, no effect on the movement between the groups was observed in the open field test. Similarly, no differences in anxiety-related behaviours, like in the time spent in the centre of the arena or in the number of contacts with the novel object during the last phase of the test, were observed. The CPP test failed to show any meaningful rewarding or aversive properties of CNO-induced activation of the Sst-neurons, while the movement rates of the groups during the conditioning trials were not different in statistically significant way. As for the withdrawal symptoms, all the mice showed the predetermined symptoms, but the test failed to show any differences between the study groups.

The neuronal tracing revealed connectivity for the adBNST Sst-neurons with brain regions involved in fear- and anxiety behaviour, social encounters, and autonomic control. In spite of this, the CNO-induced chemogenetic activation of the adBNST Sst-neurons failed to show any significant behavioural effects in the chosen paradigms for anxiety-, and reward-related behaviours, and for withdrawal symptoms. Further research is needed to dissect the Sst-subcircuitry of adBNST, both in order to verify the observed output regions, and to elucidate the role these neurons play in modification of behavioural phenotypes.

Avainsanat – Nyckelord – Keywords

somatostatin, BNST, anxiety, addiction, DREADD

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Tiivistelmä/Referat – Abstract

Pääterihmaston tyvitumake (BNST; the bed nucleus of the stria terminalis) on ollut kasvavan tutkimuskiinnostuksen kohteena viimeisten kymmenen vuoden aikana, koska sen on havaittu olevan tärkeä keskushermoston tiedonkäsittelyn solmukohta, joka ohjailee ahdistukseen, stressiin ja pelkoon liittyvää käyttäytymistä. Sen on myös todettu olevan osa riippuvuudessa toimivaa hermoverkkoa. BNST on monimutkainen ja hyvin heterogeeninen aivoalue, joka koostuu useista eri alatumakkeista ja hermosolupopulaatioista. Yksi merkittävistä BNST:n hermosolupopulaatioista on somatostatiinia ilmentävät hermosolut, jotka ovat keskittyneet BNST:n anterodorsaalille alueelle, erityisesti ovaalitumakkeeseen ja juxtakapsulaaritumakkeeseen. Näiden hermosolujen ominaisuuksia ja toimintaa tunnetaan erityisen huonosti. Tämän työn tarkoituksena on kartoittaa BNST:n somatostatiini-hermosolupopulaation hermostollisia yhteyksiä, sekä selvittää niiden osuutta BNST:hen yhdistetyn käyttäytymisen muodostumisessa, erityisesti keskittyen ahdistus-, palkkio-, ja vieroitusoirekäyttäytymiseen.

Hermosoluyhteyksien selvittämiseksi käytin AAV-vektorilla somatostatiini-tdTomato-reportterihiirilinjaan (n=2) siirrettyä cre-riipuvaisesti myristyloitua vihreää fluorensoivaa proteiinia (GFP; green fluorescent protein). Käyttäytymisroolin selvittämiseksi somatostatiini-IRES-cre-hiirikantaan siirrettiin niin ikään AAV-vektorilla cre-riippuvaisesti hM3Dq-DREADD-reseptoreita (designer receptor exclusively activated by designer drug; n=21) ja mCherry-fluorensoivaa proteiinia sisältävää kontorllirakennetta (n=20). Hiirille annettiin intraperitoneaalisesti 1,0 mg/kg klotsapiini-N-oksidia (CNO) 30 minuuttia ennen jokaista käyttäytymiskoetta designerreseptorien aktivoimiseksi. Akuutin ahdistukäyttäytymisen arviomiseksi käytin kohotettua ristisokkeloa (elevated plus maze, EPM) sekä muunneltua avoimen kentän (open field, OF) koetta, jossa keske kokeen kentälle asetetaan uusi esine. Palkkiokäyttäytymistä arvioitiin paikkaehdollistumalla (conditioned place preference, CPP), ja vieroitusoireita mallinnettiin naltreksonilla nopeutettua morfiini-vieroituskoetta (vieroitusoireissa n=9 hM3Dq, n=8 kontrolli).

Hermosolujen jäljestyskoe paljasti antreodorsaalin BNST:n somatostatiinisolujen viejähaarakkeiden kulkeutuvan aivoalueille, joiden tunnetaan olevan tekemisissä stressin, pelon ja sosiaalisten kanssakäymiseen liittyvien käyttäytymisten säätelyssä sekä autonomisten ja homeostaattisten toimintojen ohjailussa: fluorensoivia hermosäikeitä oli nähtävissä mantelitumakkeessa, hypotalamuksessa, periakveduktaalisessa harmaassa aineessa, ventraalisessa linssitumakkeessa sekä parabrakiaalisessa tumakkeessa. Käyttäytymiskokeiden osalta, DREADD-indusoitu somatostatiinineuronien aktivointi ei aiheuttanut näkyviä käyttäytymismuutoksia EPM- tai OF-kokeessa: ryhmien välillä ei ollut eroja liikeaktiivisuudessa, mutta ei myöskään ahdistukseen liitetyissä muuttujissa kuten ristisokkelon avoimissa haaroissa tai avoimen kentän keskiosassa vietetyssä ajassa. CPP-koe ei niin ikään paljastanut eroavaisuuksia ryhmien välillä. Morifiini-vieroituskokeessa kaikilla hiirillä havaittiin tyypilliset fyysiset vieroitusoireet, mutta eroja niiden määrissä tai kestoissa ei ryhmien välillä havaittu.

Tämä pro gradu –tutkimus osoittaa alustavasti, että anterodorsaalisen BNST:n alueella sijaitsevat somatostatiinineuronit hermottavat tunnettuja ahdistus- ja pelkoreaktioita sekä elimistön autonomisia vasteita sääteleviä aivoalueita. Käyttäytymiskokeet eivät kuitenkaan kyenneet osoittamaan tämän solupopulaation kemogeneettisen aktivoinnin aiheuttavan käyttäytymismuutoksia valituissa kokeissa. Näin ollen tarvitaan jatkotutkimuksia kyseisen somatostatiinihermosolupopulaation ominaisuuksien selvittämiseksi.

Avainsanat – Nyckelord – Keywords

somatostatiini, DREADD, ahdistus, riippuvuus

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CONTENTS

ABBREVATIONS

aca	anterior commisure
adBNST	anterodorsal part of the bed nucleus of the *stria terminalis*
BNST	the bed nucleus of the *stria terminalis*
BLA	basolateral amygdala
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CeA	central nucleus of amygdala
CNO	clozapine-N-oxide
CPP	conditioned place preference
CRF	corticotrophin releasing factor
CSF	cerebrospinal fluid
DREADD	designer receptor exclusively activated by designer drug
EPM	elevated-plus maze
GABA	γ-amino butyric acid
GFP	green fluorescent protei
GH	growth hormone
GI	gastrointestinal (tract)
HPA	hypothalamic-pituitary-adrenal
i.p.	intraperitoneal (administration)
IPAC	interstitial nucleus of the posterior limb of the anterior commisure
juBNST	juxtacapsular nucleus of the bed nucleus of the *stria terminalis*
LH	lateral hypothalamus
MAPK	mitogen-activated protein kinase
MDD	major depressive disorder
MeA	medial nucleus of amygdala
NAc	*Nucleus accumbens*
nNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
OCD	obsessive compulsive disorder
OF	open field
ovBNST	oval nucleus of the bed nucleus of the *stria terminalis*
p53	tumour protein 53
PAG	periaqueductal grey
PBN	parabrachial nucleus
PFC	prefrontal cortex
PTSD	post-traumatic stress disorder
PV	parvalbumin
s.c.	subcutaneous (administration)
SN	*Substantia nigra*
Sst	somatostatin
Sstr	somtostatin receptor
st	*stria terminalis*
VP	ventral pallidum
VTA	ventral tegmental area

1 INTRODUCTION

The bed nucleus of the *stria terminalis* (BNST) is a small brain area in the posterior forebrain, known to be part of the valence processing circuitry of the brain (Somerville et al. 2010; Kim et al. 2013; Calhoon and Tye 2015). The BNST has long been known to mediate stress-related, defensive, and social behaviours, but recent advances in genetic and neuron-controlling tools, such as optogenetics and transgenic animals, have brought forth new pieces of evidence of the BNST's potential role in debilitating stress-related neuropsychiatric disorders, like generalized anxiety disorder, and addiction (Erb et al. 2001; Kim et al. 2013). This has raised a new interest in researching the BNST in humans after several decades of retirement (Avery et al. 2014; Straube et al. 2007). It is also an intriguing brain area with significant sexual dimorphism, which has gained the BNST some societal relevance through studies showing that the dimorphic characteristics are reversed in transgender people (Allen and Gorski 1990; Zhou et al. 1995). The BNST is regionally highly heterogeneous, and well known as a significant source of corticotrophin releasing factor (CRF) expressing neurons, which have been extensively studied for their role in initiating stress responses (Bota et al. 2012; Partridge et al. 2016). BNST also harbours a major group of somatostatin (Sst) expressing neurons. While the presence of these Sst-neurons has been known for years, virtually no research studying their neuronal actions or their role in regulation of behaviour has been published.

This work will first review the current knowledge on both Sst and the BNST. While Sst is an important active substance working both in the central nervous system and elsewhere in the system, mainly being expressed in the gastrointestinal (GI) tract and the pancreas, the review will concentrate on its role in the central nervous system. The work will then continue to describe and discuss the experiments aiming to characterise the anatomical connectivity of Sst-neurons in the anterodorsal part of the BNST, and their role in neuropsychiatrically relevant behavioural phenotypes.

2 SOMATOSTATIN

In 1972, Brazeau et al. discovered and purified a peptide substance that inhibits the secretion of growth hormone in sheep hypothalamus. This hormone, today known as somatostatin (Sst, also SOM), is widely expressed in the mammalian system, being secreted as a hormone from the pituitary and the GI-tract, and expressed as a neuropeptide in neurons in the central nervous system (Guillemin 2008; Rai et al. 2015). It inhibits the release of gastric peptides and hormones in GI-tract and in pancreas. Synthetic analogues of Sst (such as octreotide, lantreotide, pasitreotide) are widely used as drugs to treat acromegaly, the state of excess secretion of growth hormone, but also in different kinds of neuroendocrine, and paracrine tumours. The exhaustive summary of somatostatin's role throughout the body is not in the scope of this review, and therefore the following discussion is concentrated on Sst in the brain.

2.1 Somatostatin and its receptors

Sst is a cyclic polypeptide hormone synthesized as a larger prohormone, pre-prosomatostatin (Wynick et al. 1989; Patel 1999). Through enzymatic cleavage from the C-terminal, the two bioactive forms of Sst are produced: the shorter, a 14 amino-acid-long peptide with a disulphide bridge between the two cysteine residues (SS14), is the more abundant form in the nervous system, while the other, a 28 amino-acid-long congener of the shorter peptide, elongated from the N-terminal (SS28), is prevalent in the peripheral tissues. In this work, the word somatostatin and abbreviation Sst are used to describe collectively both active forms. In the nervous system, Sst is synthesized mainly in neurons, varying in their location and morphology, as described later, but also in a lesser extent in glial cells (Davidson and Gillies 1993; Liguz-Lecznar et al. 2016). In neurons, Sst is stored in dense-core vesicles that are trafficked widely into dendrites, axon and neuron soma, and is released in response to high frequency repetitive action potentials (Baraban and Tallent 2004; Ludwig and Leng 2006; Zhang et al. 2010). The release is usually slower than that of the classical neurotransmitters, due to the larger size of the peptide. The duration of the action is also longer, since there are no specialized uptake mechanisms for Sst, and therefore the peptide remains in the extracellular space for longer periods.

Sst exhibits inhibitory effects, and is especially known to block secretion of several hormones and other substances (Theodoropoulou and Stalla 2013). Namely, it suppresses the secretion of (1) growth hormone (GH) and prolactin in the pituitary, (2) glucagon and insulin in the pancreas, (3) and several other peptides and hormones in the gastrointestinal tract and in the kidneys (Brazeau et al. 1973; Bloom et al. 1974; DeVane et al. 1974; Gomez-Pan et al. 1976). In the nervous system, Sst works as an inhibitory neuropeptide, causing hyperpolarisation and decreased neurotransmitter release in the target neurons (Viollet et al. 2008). Additionally, Sst is known to have antiproliferative effects, especially in tumour cells, by being able to arrest the cell cycle and to induce apoptosis (Cordelier et al. 1997; Thangaraju et al. 1999). Sst is known to be an evolutionarily conserved peptide throughout the vertebrates, making the translation of the results in animal studies into clinical applications more viable (Gahete et al. 2010). However, the interspecies similarity of the effects of Sst are difficult to predict due to differences in receptor types and signal cascades (Enjalbert et al. 1982; Giordano et al. 2007).

The physiological actions of Sst are mediated through five structurally similar receptor proteins, known as $Sstr_1$ to $Sstr_5$, with differing expression patterns and distinct pharmacological properties (Table 1; Bruno et al. 1992; Patel 1999). All five receptors are G-protein coupled, and share some of the metabotropic signalling cascades, like the inhibition of adenylyl cyclase (AC), and modulation of mitogen-activated protein kinase (MAPK). The receptors also have subtype specific intracellular interaction pathways, making subtype-selective specialisation possible.

The inhibition of secretory action is usually mediated through the inhibition of exocytosis, by Sst inhibiting AC and reducing the cyclic adenosine monophosphate (cAMP) formation (Figure 1; (Heisler et al. 1982). The receptors are also linked with several subsets of K^+ ion channels, through which they cause cell membrane hyperpolarisation (Wang et al. 1989; Sims et al. 1991). The hyperpolarisation also decreases Ca^{2+} inflow through the inhibition of voltage-sensitive Ca^{2+} channels. The cell cycle arrest is induced by the activation of MAPK, while the activation of tumour protein 53 (p53) triggers apoptosis, an effect mostly credited to $Sstr_3$-linked pathways (Cordelier et al. 1997; Thangaraju et al. 1999; Theodoropoulou and Stalla 2013).

Table 1. Properties of somatostatin receptors. Both the intracellular effects, and the expression sites in the brain represent only the most major systems described in the literature, as comprehensive listing of all findings is outside of the scope of this review. Legend: ↑ increased activity/level, ↓ decreased activity/level, = no shown effect, blank denotes no knowledge. Adapted from Patel 1999; Liguz-Lecznar et al. 2016; Theodoropoulou and Stalla 2013.

	$Sstr_1$	**$Sstr_2$**	**$Sstr_3$**	**$Sstr_4$**	**$Sstr_5$**
G-protein coupling	$G_{i/o}$	$G_{i/o}$ $G_{q/11}$	$G_{i/o}$ $G_{q/11}$	$G_{i/o}$	$G_{i/o}$ $G_{q/11}$
Affinity for Sst14 and Sst28	14 = 28	14 = 28	14 = 28	14 = 28	14 < 28
cAMP	↓	↓	↓	↓	↓
cGMP	↑	↑			↓
Adenylyl cyclase	↓	↓	↓	↓	↓
K^+ channels	↑	↑	↑	↑	↑
Ca^{2+} channels	↓	↓	=		↓
MAPK	↑	↓/↑	↑/↓	↑	↓
p53		↑	↑		
Major expression in the brain	amygdala, cortex, hippocampus, hypothalamus	amygdala, cortex, hippocampus, hypothalamus	amygdala, cerebellum, hippocampus, olfactory bulb, striatum	amygdala, cerebellum, hippocampus, olfactory bulb, striatum	hypothalamus, preoptic area

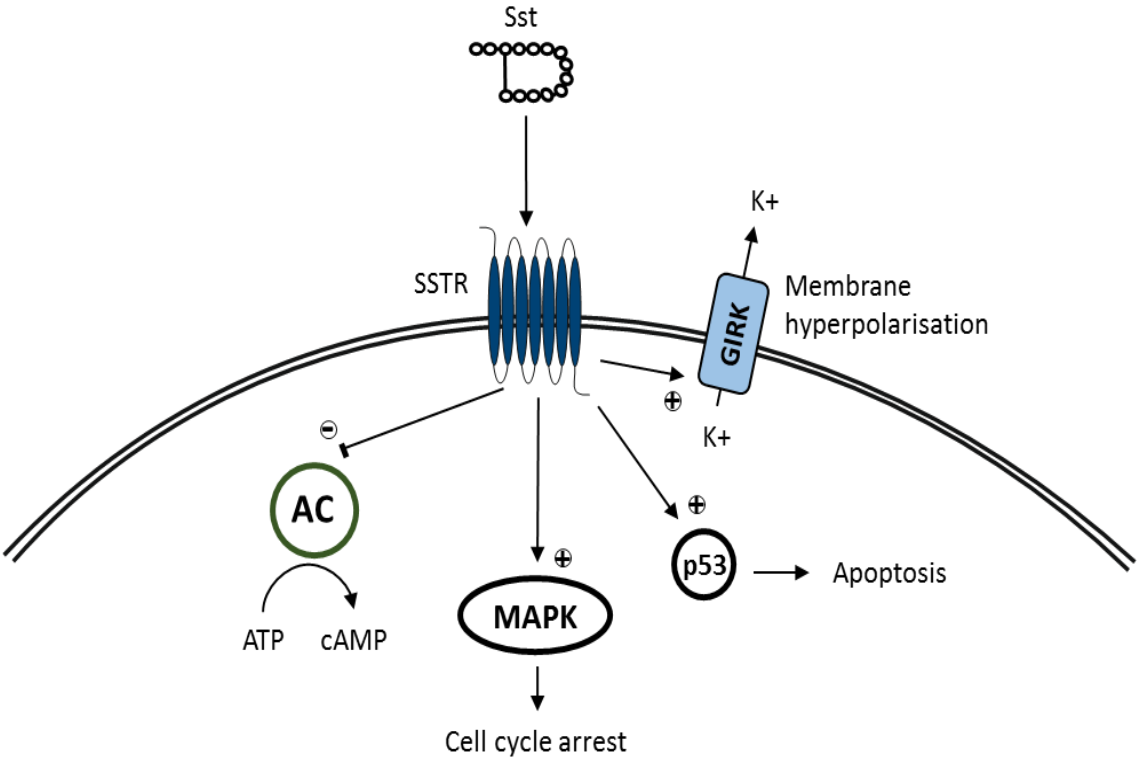


Figure 1. Some of the intracellular pathways in Sst-signalling. Sst inhibits adenylyl cyclase (AC), reducing the levels of cyclic AMP (cAMP), a widely active secondary messenger, also needed for induction of exocytosis. Sst can also activate the MAP kinase (MAPK) and tumour protein 53 (p53), which are linked to Sst-induced cell cycle arrest and apoptosis, respectively. Sst also activates several potassium channels, including the G protein-coupled inwardly-rectifying potassium channel (GIRK), which lead into membrane hyperpolarization through potassium (K^+) outflow.

2.2 Somatostatin in the central nervous system

Somatostatin-expressing neurons (Sst-neurons) are widely distributed in the mammalian central nervous system, and are, for example, found in great abundance in the forebrain areas like cortex, hypothalamus, amygdala, and the extended amygdala structures, but also in the brainstem and the spinal cord (Giehl and Mestres 1995; Saha et al. 2002; Tomioka et al. 2005; Viollet et al. 2008). Being one of the main subclasses of the cortical interneurons, Sst-neurons have been a target for scientific interest for decades, revealing significant heterogeneity and variability in their properties between, and within, the brain structures. Even though it is true that most research on Sst in the brain is based on studies made with animal models (also applicable to most of the research referenced in the

following), the expression of Sst and its receptors in humans is very similar with rodents (Reubi et al. 1986; Mengod et al. 1992). This is especially true in the cortex, the amygdaloidal structures, and the brainstem. The following will outline properties of Sst-neurons, brain regions with high expression of Sst-neurons, and discuss some of the known neuropsychiatric disorders linked with changes in the Sst-system.

2.2.1 Somatostatin-expressing neurons

Sst-neurons are a heterogeneous group of neurons, with varying electrophysiological, morphological, and neurotransmitter expression profiles (Liguz-Lecznar et al. 2016). Due to the diversity of the Sst-neurons, it might be problematic to treat them as a unitary group of neurons, and for that, several attempts on further classifying the Sst-neurons throughout the brain have been made (Wang et al. 2002; Ma et al. 2006; Sosulina et al. 2006; McGarry et al. 2010). Sst-neurons are most often classified by either their electrophysiological or molecular properties. The electrophysiological profiles of the Sst-neurons seem to vary, to some extent, between different brain regions. The majority of the cortical Sst-neurons show classical accommodating responses, but subsets of burst-accommodating, non-accommodating, and irregular spiking Sst-neurons have also been identified (Wang et al. 2002; Wang et al. 2004). It has also been shown that in γ-aminobutyric acid (GABA) releasing Sst-neurons, GABA and Sst release contribute to different kinds of postsynaptic signals (Tang and Augustine 2015). In study with claustrum Sst-neurons, the acute inhibitory postsynaptic currents were GABA-dependent, while Sst caused prolongation of action potential duration.

Sst-neurons often co-express GABA, which is natural seeing the potential synergistic effects of two transmitters with inhibitory action (Viollet et al. 2008). Besides that, Sst-neurons have been shown to have co-expression with several other neuropeptides, like corticotrophin releasing factor (CRF), neuropeptide Y (NPY), and neuronal nitric oxide synthase (nNOS), and with calcium-binding proteins, like parvalbumin (PV), calbindin, and calretinin. There are also data showing co-expression of Sst and dopamine in the hypothalamus, and of Sst and tyrosine hydroxylase, a commonly used marker for dopamine neurons, in the midbrain (Dulcis et al. 2013; Forss M, unpublished data 2017). Like with the electrophysiological profiles, the co-expression of Sst and other transmitters

and proteins varies greatly between the brain areas, and different subtypes seem to be typical for different anatomical regions: for example, in the rodent frontal and visual cortices, Sst- and calretinin have not been shown to overlap, whereas in sensorimotor cortex, 40 % of Sst-neurons were shown to co-express calretinin (Kubota et al. 1994; Halabisky et al. 2006). Seeing the high variety of co-expressing neuropeptides and transmitter, and the above-mentioned observation on Sst and transmitter being able to cause different kinds of postsynaptic signals, it would be imprudent to group all the neurons expressing Sst into one category.

In the cortex, Sst is co-expressed with GABA in inhibitory neurons, constituting 20 – 30 % of all GABA neurons in the neocortex (Tomioka et al. 2005; Uematsu et al. 2008). Out of these, interneurons called Martinotti cells are the predominant cell type. They are mostly found on the layers 2/3 and 5, innervating the apical dendrites of the pyramidal neurons of the same cortical layers in the layer 1(Kawaguchi 1993; Ma et al. 2006). A smaller group of Sst-interneurons, known as the nest- and small-basket cells, has also been identified in the cortical layers 4 and 5. The Sst-interneurons actively participate in integration of sensory inputs, and in formation of synchronised oscillations together with PV-interneurons, and are implicated in experience-dependent and *de novo* neuronal plasticity, thus playing a role in reshaping neural networks (Kuki et al. 2015; Scheyltjens and Arckens 2016; Tang et al. 2014). Some of the cortical Sst-neurons are also known to send projections to other cortical regions (cortico-cortical projections) and to the striatum (Tomioka et al. 2005; Rock et al. 2016) These neurons constitute only a small fraction of the cortical Sst-neurons, but have been shown to participate in control of behaviour: it was recently shown, that optogenetic modulation of the cortico-striatal Sst-neuron projection affected the locomotor activity of mice (Melzer et al. 2017).

Like in the cortex, locally innervating and projecting Sst-neurons have been identified in the amygdala (Saha et al. 2002; Sosulina et al. 2006). The Sst-interneurons innervate the local principal neurons and other interneurons in a distal dendrite preferring manner, and are, in close co-operation with PV-interneurons, strongly involved in controlling fear related behaviours in rodents (Muller et al. 2007; Li et al. 2013; Wolff et al. 2014). The other subgroup of amygdala Sst-neurons are projection neurons, which form two distinct

projection systems. The first one emerges from the lateral amygdala, innervating hypothalamus, entorhinal cortex, and more locally the other amygdala nuclei, except for central nucleus (CeA; McDonald et al. 2012; McDonald and Zaric 2015). From the CeA originates the other Sst-projection system, sending outputs to the bed nucleus of the *stria terminalis* (BNST), and through it to the brainstem region, like the parabrachial nucleus (PBN) and the nucleus of solitary tract (Sosulina et al. 2006; Viollet et al. 2008). Also in the CeA, a set of predominantly Sst-expressing neurons sending projection to periaqueductal grey (PAG), and to paraventricular thalamic nucleus has been identified (Penzo et al. 2014). These GABAergic neurons are shown to be an important contributor to fear conditioning in mice, going through robust synaptic plasticity during the behavioural process.

In addition to being a target for the amygdala Sst-projections, the BNST harbours in itself a significant population of Sst-neurons (Finley et al. 1981; Moga et al. 1989; Walter et al. 1991; Nguyen et al. 2016). They are most concentrated in the oval and the juxtacapsular nuclei of the BNST, but also seen slightly scattered in the anteromedial and the anterolateral nuclei. These Sst-neurons are known to locally inhibit other neurons in the BNST, but apart from the projections to the PBN, no studies on their output profiles outside the BNST have been published (Moga et al. 1989; Magableh and Lundy 2014; Xu et al. 2016).

One more major Sst-projecting systems is also found in the hypothalamus, more precisely in periventricular and paraventricular nuclei of hypothalamus (Dierickx and Vandesande 1979). The Sst-neurons in hypothalamus project to the median eminence, affecting the pituitary cells through the capillaries of the portal vasculature, as well as to several parts of the limbic system and the midbrain areas, like the habenula, hippocampus, and *substantia nigra* (Viollet et al. 2008).

2.2.2 Somatostatin in neuropsychiatric disorders

Several studies have shown changes in Sst-system in a variety of neurological and neuropsychiatric disorders (Lin and Sibille 2013). The best known dysfunction of neuronal system with a link to Sst is acromegaly, the excess production of growth

hormone often caused by pituitary adenoma, to which Sst analogues have been the main clinical intervention for several years (Yen et al. 1974; Giustina et al. 2000). This endocrinological connection is well established, but the relationship between Sst and the several neuropsychiatric disorders remains elusive (Lin and Sibille 2013).

As discussed earlier, high abundance of Sst-neuron is found in fear- and stress-related brain areas, the amygdala and the BNST (Sosulina et al. 2006). Therefore, it is not a surprise that Sst has been linked with stress- and anxiety-related behaviours (Kluge et al. 2008; Albrecht et al. 2013). Direct infusions of Sst and its analogues to amygdala have been shown to exhibit anxiolytic effects, and the activation of Sst-system is known to cancel out the anxiogenic effects of CRF (Brown et al. 1984; Engin and Treit 2009; Yeung et al. 2011). Increased Sst release, and Sstr upregulation have also been reported in response to stressors, and the disinhibition of Sst-interneurons has been shown to result in anxiolysis (Nanda et al. 2008; Butler et al. 2012; Fuchs et al. 2016). These data, among others not mentioned here, suggest that Sst-signalling is inherently anxiolytic, and that the Sst-systems are recruited during the stress and in anxious states to antagonise the anxiogenic effects of the CRF-signalling. Interestingly, and contrarily to the aforementioned, a research showed that increased Sst release and increased $Sstr_{2/4}$: dopamine Drd2 –ratio on rat hypothalamic CRF-neurons increases anxiety-linked behaviour and the amounts of stress hormone corticosterone in the cerebrospinal fluid (CSF; Dulcis et al. 2013). This can still be, at least partly, explained by decreased inhibitory signalling on the CRF-neurons caused by the downregulation of the inhibitory Drd2-autoreceptors, but is still to show that the anxiolytic effect of Sst-release is not straightforward.

Tripp et al. (2011) showed that the expressions of Sst and its pre-pro form are reduced in the anterior cingulate cortex in *post mortem* samples of human patients with major depressive disorder (MDD). Similar findings have been reported in different brain areas, like the prefrontal cortex (PFC), and the amygdala (Sibille et al. 2011; Guilloux et al. 2012). These findings match the results of earlier studies, showing decreased Sst-levels in the CSF during depressive episodes (Agren and Lundqvist 1984; Molchan et al. 1993). Both, the Sst-system dysfunctions, and the occurrence of MDD, have been found to be

more prevalent in female patients in several cohorts, which has been used as an indirect proof for the relation of Sst and depressive disorders by some researchers (Sibille et al. 2011; Tripp et al. 2011; Guilloux et al. 2012; Lin and Sibille 2013).

Similar findings to MDD have been reported in patients with other neuropsychiatric disorders. In schizophrenia patients CSF Sst is also decreased, as is Sst-RNA expression in PFC (Reinikainen et al. 1990; Guillozet-Bongaarts et al. 2014). In addition, the number and density of Sst-neurons is reduced in hippocampus and entorhinal cortex (Konradi et al. 2011; Wang et al. 2011). Similar decreases in the Sst-neuron densities in the limbic systems, as well in Sst-RNA expression levels in PFC are seen in patients with bipolar disorder (Konradi et al. 2011; Sibille et al. 2011; Wang et al. 2011).

One of the most constant Sst-related findings is the loss of Sst-interneurons in epilepsy (Sloviter 1987; Houser 2014). Several kinds of interneurons are shown to be affected, but the loss of, and the reorganisation of the remaining Sst-interneurons in the dentate gyrus are recurrently reported in the epileptic patients, and shown to be true in the most of the models of epilepsy as well (de Lanerolle et al. 1989; Robbins et al. 1991; Peng et al. 2013; Houser 2014). The reasons behind the Sst-interneuron selectivity are not yet known, but the findings have lead into some trials investigating Sst and its analogues as anticonvulsant medications, with some promising preclinical results (Vezzani et al. 1991; Kozhemyakin et al. 2013).

Changes in the Sst-system like the ones described in the cases of neuropsychiatric disorders have been demonstrated also in neurodegenerative diseases (Lin and Sibille 2013). In Alzheimer's disease, a strong correlation between the changes in Sst-system and cognitive capabilities of the patients have been shown (Epelbaum et al. 2009). In Alzheimer's disease patients, Sst-RNA expression and immunoreactivity is decreased, especially in the hippocampus, and widely in cortex (Davies et al. 1980; Candy et al. 1985; Dournaud et al. 1994). Moreover, the CSF Sst-levels have been shown to be decreased, both in Alzheimer's disease and in Parkinson's disease patients with dementia (Beal et al. 1986; Bissette et al. 1986). Recent studies have also investigated the role of Sstrs in downregulated Sst-signalling, finding that the hypermethylation of Sstr4 did not correlate

with the progression or symptomatology of the Alzheimer's disease, but implicating the loss of Sstr2 function in the dysfunction of noradrenergic system in the Alzheimer's disease (Grosser et al. 2014; Adori et al. 2015).

To sum up, it has been shown that Sst retains, to some extent, anxiolytic and anticonvulsant properties (Brown et al. 1984; Tallent and Qiu 2008; Kozhemyakin et al. 2013). Sst has also been implicated in several neuropsychiatric disorders (Table 2). While it is true that the evidence on the correlation of Sst-related deficits and the neuropsychiatric disorders keeps cumulating, and the research papers showing the correlation are readily cited, it remains questionable, if the Sst-system itself is the cause behind the disorders. Because Sst is widely expressed in neurons throughout the brain, and the Sst-neurons constitute almost roughly one third of the cortical interneurons, the decreases in the Sst-levels could be only a secondary biomarker of wider deficiencies in: for example, disturbances in the development of all cortical interneurons – known to be present in schizophrenia – would certainly affect also the Sst-interneurons, which in turn could lead into decreased Sst-RNA expression, as well as in lower Sst-levels in CSF (Volk and Lewis 2014). While the evidence on the correlation is undeniable, there is still room for further exploration to elucidate the role of Sst in neuropsychiatric disorders.

Table 2. Neuropsychiatric conditions where changes in Sst-system are implicated.

Disorder	Findings	Reference
Anxiety	Amygdala infusion of Sst reduces anxiety	Butler et al. 2012
	Sst ↑ Sstr ↑ in stress	Engin and Treit 2009; Nanda et al. 2008
Mania	CSF Sst ↑	Sharma et al. 1995
OCD	CSF Sst ↑	Altemus et al. 1993
PTSD	CSF Sst ↑ CSF Sst =	Bremner et al. 1997 Sautter et al. 200)
Depression	CSF Sst ↓	Agren and Lundqvist 1984
	Sst expression ↓ - *prefrontal cortex* - *cingulate cortex* - *amygdala*	 Sibille et al. 2011 Tripp et al. 2011 Guilloux et al. 2012
Schizophrenia	CSF Sst ↓	Reinikainen et al. 1990
	Sst-RNA ↓ in - *prefrontal cortex*	 Guillozet-Bongaarts et al. 2014
	Sst-neuron ↓ in - *hippocampus* - *entorhinal cortex*	 Konradi et al. 2010 Wang et al. 2011
Bipolar disorder	Sst-RNA ↓ in - *prefrontal cortex*	 Sibille et al. 2011
	Sst-neuron ↓ in - *hippocampus*	 Konradi et al. 2010
Epilepsy	Sst-neurons ↓ - *hippocampus*	 de Lanerolle et al. 1989
	Sst-neuron reorganisation - *hippocampus*	 de Lanerolle et al. 1989
Alzheimer's disease	CSF Sst ↓	Bissette et al. 1986
	Sst-RNA ↓ - *cortex* - *hippocampus*	 Candy et al. 1985 Dournaud et al. 1994)
Parkinson's disease	CSF Sst ↓	Beal et al. 1986

3 THE BED NUCLEUS OF THE STRIA TERMINALIS

As mentioned earlier, the most significant Sst-neuron populations apart from the cortical interneurons have been observed in the hypothalamus, in the amygdala, and in the so called extended amygdala structures (Moga et al. 1989; Saha et al. 2002; McDonald et al. 2012). The extended amygdala is a concept of a loop-like brain structure connecting the CeA and the medial nucleus of the amygdala (MeA), and the bed nucleus of the *stria terminalis* (BNST; Heimer 2003). The BNST has been a target of expansive interest in neuroscience field for several decades, and increasingly so during the last ten years due to its observed relevance in human stress-related psychiatric disorders, and the development of new technologies to study the BNST in detail (Lebow and Chen 2016). The BNST is a small forebrain structure, uniquely located as a connective hub between the cortical and the subcortical structures, integrating sensory, memory- and affection-related information as a part of the valence processing circuitry (Calhoon and Tye 2015). It is an important structure in stress-related, social, defensive, and reproductive behaviours. This is also reflected in heterogeneous receptor expression in the BNST (Figure 4), indicating a wide variety in the nature of the neuronal inputs. The following chapter outlines the anatomy and connectivity of the rodent BNST, introduces the human BNST together with some translational aspects, and discusses some of the known behavioural roles of the brain region.

3.2 The anatomy and the connectivity of the rodent BNST

In rodents, the BNST is situated ventral to the septal nuclei and the lateral ventricles, occupying a small area dorsal and ventral to the anterior commissure, and ventromedial to the striatum, next to the internal capsule (Figure 2; Lebow and Chen 2016). Thus, it is perfectly positioned for its role of an information integrator: the BNST receives various inputs from cortical areas and the olfactory bulb, relaying sensory information, from the limbic system, such as the amygdala and the hippocampus, and from the midbrain areas related to reward, social interactions and stress control, like the VTA, the raphe nuclei, and the *locus coeruleus*. The BNST then sends information back to these structures to form a feed-back loop, but has also large outputs to brain areas controlling homeostasis,

such as the hypothalamus, the septal nuclei, the nucleus of the solitary tract, and the parabrachial nucleus (PBN).

In rodents, the BNST is divided into several subdomains, the number of which vary between 12 and 18, depending on the author. This work is based on widely-accepted division of Bota et al. (2012), where 12 distinct regions with differing connectivity and chemoarchitectural properties are acknowledged. The common main division of the BNST is into its anterior and posterior parts. The anterior BNST includes the anterolateral, anteromedial, oval, fusiform, juxtacapsular, rhomboid, dorsomedial, ventral and magnocellular nuclei, while the posterior part is divided into only three subregions, the principal, intrafascicular, and transverse nuclei.

The oval nucleus of BNST is, as the name implies, an oval shaped area between the internal capsule and the lateral ventricle, dorsal to the anterior commissure (Dong et al. 2001; Bota et al. 2012). It receives projections from the amygdala, especially from the CeA, dopaminergic projections from the VTA, noradrenergic projections from the *locus coeruleus*, and is known to be innervated by serotonin- and dopamine-neurons from the dorsal raphe nuclei (Dong et al. 2001; Marcinkiewcz et al. 2016). Its GABAergic neurons are known to densely innervate locally, especially the neighbouring anterolateral and anteromedial nuclei of the BNST (Larriva-Sahd 2006; Jennings et al. 2013; Kim et al. 2013; Turesson et al. 2013). Also, several kinds of neurons projecting to the amygdala, VTA, hypothalamus, and PBN have been identified. Of the BNST nuclei, it is in the oval BNST where the most abundant expression of Sst-neurons is observable (Figures 3 and 7; Moga et al. 1989; Bota et al. 2012). Some of these Sst-neurons are shown to project to the PBN (Moga et al. 1989; Magableh and Lundy 2014).

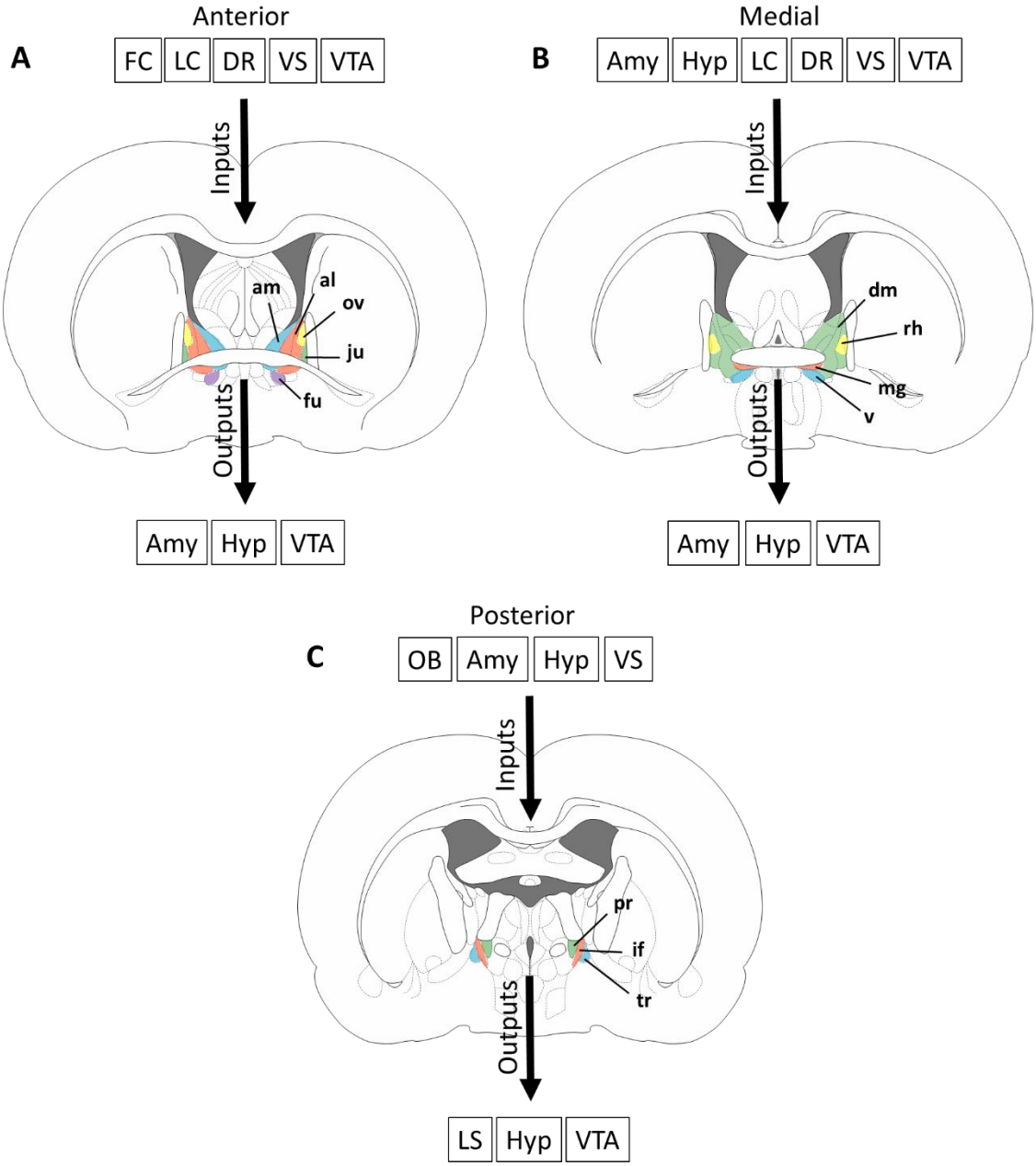
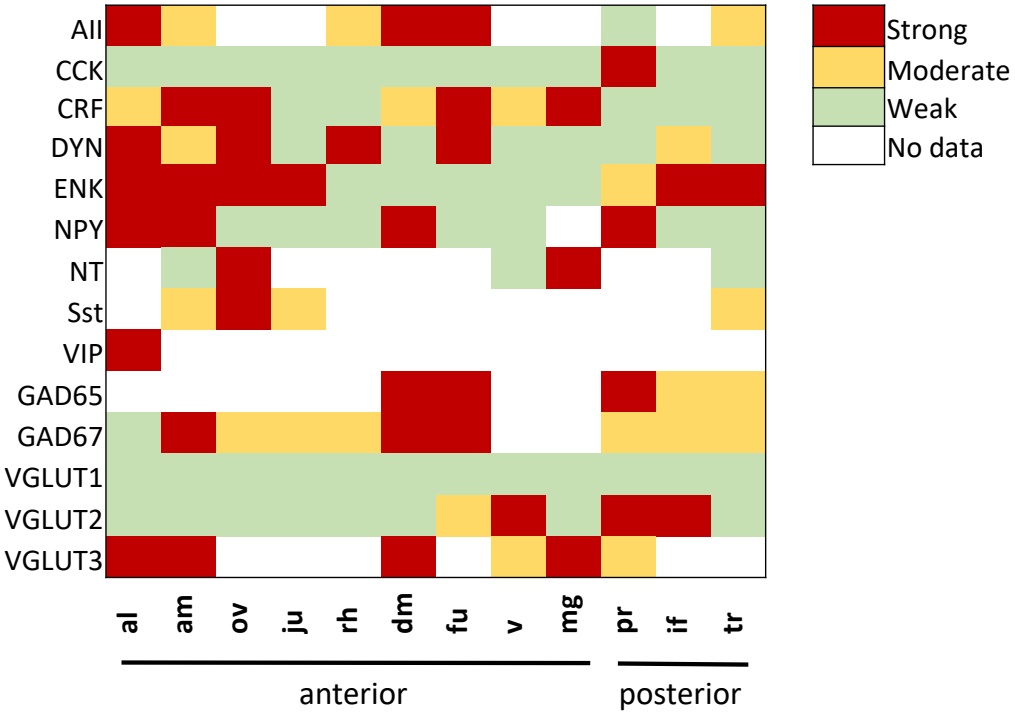


Figure 2. Subdivisions and the main connections of the rodent BNST. **A.** The most anterior region of BNST consists of anteromedial (am, blue), anterolateral (al, red), oval (ov, yellow), juxtacapsular (ju, green), and fusiform nuclei (fu, purple). **B.** Still considered as part of the anterior BNST, the medial-most part of the BNST includes the dorsomedial (dm, green), rhomboid (rh, yellow), magnocellular (mg, red), and ventral nuclei (v, red). **C.** The posterior BNST consists of of principle (pr, green), interfascicular (if, red), and transverse nuclei (rh, blue). The schematic also details the main input and output connections of each of the three areas. The connectivity of the rodent BNST has been broadly described by Dong and Swanson in a series of papers using Phaseolus vulgaris-leucoagglutinin neuronal tracing method, reviewed by Bota et al. 2012; Lebow and Chen 2016. *Amy – amygdala, DR – dorsal raphe, FC – frontal cortex, Hyp – hypothalamus, LC – locus coeruleus, OB – olfactory bulb,VS – ventral subiculum, VTA – ventral tegmental area.*

The anterolateral BNST, situated ventral to the oval nucleus, is a large integrative hub of many neuronal outputs and inputs, and heavily innervating the adjacent BNST nuclei (Dong and Swanson 2004a). It receives serotonergic projection from the dorsal raphe, and is connected to the hypothalamic-pituitary-adrenal (HPA) axis negative feedback loop by its outputs to the paraventricular hypothalamus, and by glutamatergic inputs from the ventral subiculum. It is also connected to the autonomic control system through the outputs to the ventral amygdala, the periaqueductal grey (PAG), and the PBN, as well as to the somatomotor system with connections to the VTA, the *nucleus accumbens* (NAc), and the *substantia innominata*.



Legend: al – anterolateral BNST, AII – angiotensin I, am – anteromedial BNST, GAD65 – glutamate decarboxylase 65, GAD67 – glutamate decarboxylase 67, CCK – cholecystokinin, CRF – corticotrophin releasing factor, dm – dorsomedial BNST, DYN – dynorphin, ENK – encephalin, fu – fusiform BNST, if – interfascicular BNST, ju – juxtacapsular BNST, mg – magnocellular BNST, NPY – neuropeptide Y, NT – neurotensin, ov – oval BNST, pr – principle BNST, rh – rhomboid BNST, Sst – somatostatin, tr –transverse BNST, v – ventral BNST, VIP – vasoactive intestinal peptide, VGLUT1 – vesicular glutamate transporter 1, VGLUT2 – vesicular glutamate transporter 2, VGLUT3 – vesicular glutamate transporter 3.

Figure 3. Subpopulations of neuropeptides and transporter proteins in the BNST. BNST is known to be largely GABAergic, but it expresses wide variety of neuropeptides. The heatmap is adapted from collated *in situ* hybridisation and immunostaining data by Moga et al. 1989; Bota et al. 2012; Lebow and Chen 2016.

Despite their distinct locations, the juxtacapsular and the fusiform nuclei of the BNST have been shown to largely innervate the same neural systems (Dong et al. 2000; Dong et al. 2001; Larriva-Sahd 2004). The neurons from the fusiform nucleus, located ventral to the anterior commissure, project to the CeA, the PVN, the NAc, and the PAG, while the juxtacapsular nucleus, situated right next to the internal capsule, ventral to the oval BNST, sends projections to the CeA and the basolateral amygdala (BLA), *substantia*

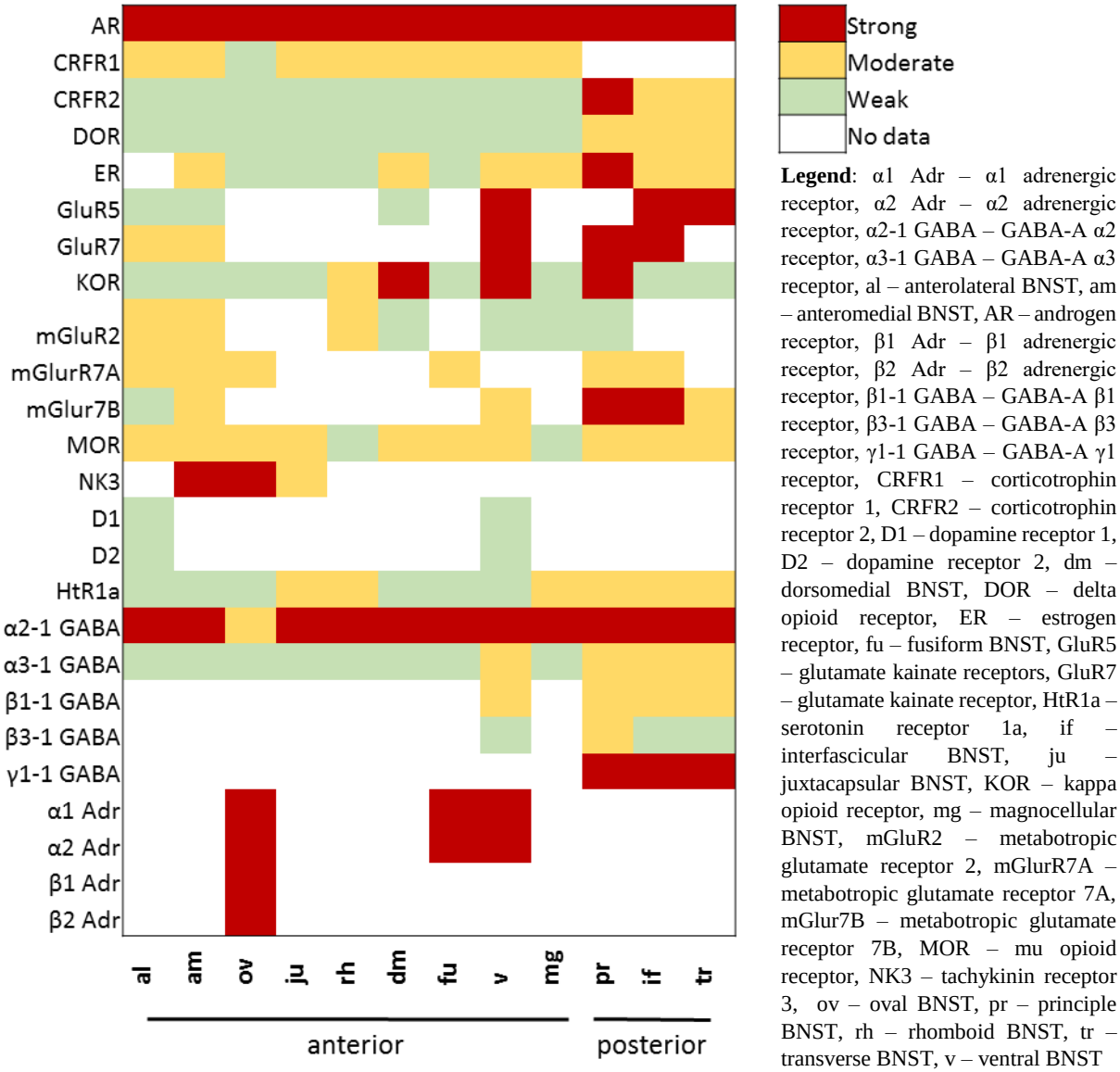


Figure 4. Subtypes of receptors in the BNST. The expression of receptors indicates, to some extent, the types of innervation BNST receives, either from the other BNST nuclei, or from the other brain areas. The heatmap is adapted from collated *in situ* hybridisation and immunostaining data by Bota et al. 2012; Lebow and Chen 2016.

nigra, and the dorsal raphe. The juxtacapsular BNST has also been shown to harbour a significant population of Sst-neurons (Moga et al. 1989).

The medial regions of the BNST harbour significant connections to the hypothalamus: the anteromedial nucleus has direct contacts to the paraventricular nucleus of the hypothalamus (PVN), while the dorsolateral BNST has been shown to have wide connectivity with the whole hypothalamus (Dong and Swanson 2006a; Dong and Swanson 2006b). In addition to the hypothalamus, the anteromedial BNST is connected with other homeostasis and autonomic control areas, like the vagal projections and the brainstem. On the other hand, the dorsomedial nucleus receives many inputs from several amygdala nuclei, the hippocampus and the frontal cortical regions, indicating a role more in the stress- and fear-related behaviours.

The posterior BNST nuclei together receive major inputs from the amygdala, especially the MeA and CeA ((Dong and Swanson 2004b). They send outputs back to the MeA, and are also strongly connected with the hypothalamic and the mesolimbic structures associated with social and reproductive behaviours, like the septal nuclei, hypothalamus, VTA and pallidum. Reflecting the reproductive behaviour-linked connections, the posterior BNST nuclei have rich expression of androgen and estrogen receptors (Hines et al. 1992; Campi et al. 2013; Janitzky et al. 2014). This is in line with the observations showing that the posterior part of the BNST is most prone to sexually dichotomous changes (Laflamme et al. 1998). In addition to the oval and the juxtacapsular BNST, the transverse nucleus in the posterior BNST seems to contain a group of Sst-neurons near the internal capsule (Moga et al. 1989)

3.2 The human BNST and translational aspects

The human BNST is situated in the ventral forebrain, roughly at the level of the anterior commissure, bordered by the internal capsule and the lateral septal nuclei (Walter et al. 1991; Lebow and Chen 2016). It can be separated into three different subdivisions: lateral, central, and medial. The divisions are grouped together on the dorsal side of the anterior commissure (Figure 5A). Part of the BNST can be seen ventral to the commissure, and is therefore at times called the ventral subdivision. Declaring this naming invalid,

histological evidence show that the region is indistinguishable from the central division, and therefore the ventral division is most likely not its own subregion (Walter et al. 1991).

The lateral division of the BNST is situated next to the internal capsule (Walter et al. 1991). The subdivision has typically a high occurrence of neuropeptide Y (NPY) expressing neurons and dense patterns of NPY-positive neuronal fibres. Also, fibres with substance P and synaptophysin immunoreactivity are often observed, but very few, or no neurons or varicosities expressing Sst or neurotensin (NT). Contrary to the lateral division, the central part of the BNST is characterised by strong Sst-immunoreactivity, both in the somas and in the neuronal fibres. Other extensively observed neuropeptides are enkephalin, and NT, with moderate innervation of vasoactive intestinal peptide (VIP) positive fibres. The medial BNST shows mostly strong substance P and NPY positive nerve fibres, in addition to moderate presence of Sst and enkephalin. Interestingly, Walter et al. reported that the neurons in the medial subdivision did not have immunoreactivity with any of the major neuropeptides they used in their study. The described immunohistochemical properties are summarized in Figure 5B.

In humans, three distinct connective pathways in the BNST have been identified (Krüger et al. 2015). The posterior bundle connects the BNST with the lateral amygdala and the cortical regions of the temporal lobe, by following the *stria terminalis* around the thalamus, while the ventral bundle follows the *ansa peduncularis* around the internal capsule to connect with the MeA and hypothalamus. Anterior pathway runs through the NAc and caudate nucleus to the prefrontal and the orbitofrontal cortices. While these three bundles are shown to be constant across individuals, there are many known brain areas connected with the BNST without consistent pathways observable with modern imaging tools (Avery et al. 2014; Krüger et al. 2015; Torrisi et al. 2017). These areas include the thalamus, the pallidum, the hippocampus, the habenula, and especially the brainstem structures. Because the structural connectivity studies in human are largely based on the use of magnetic resonance imaging (MRI), there are limitations to the level of acquired

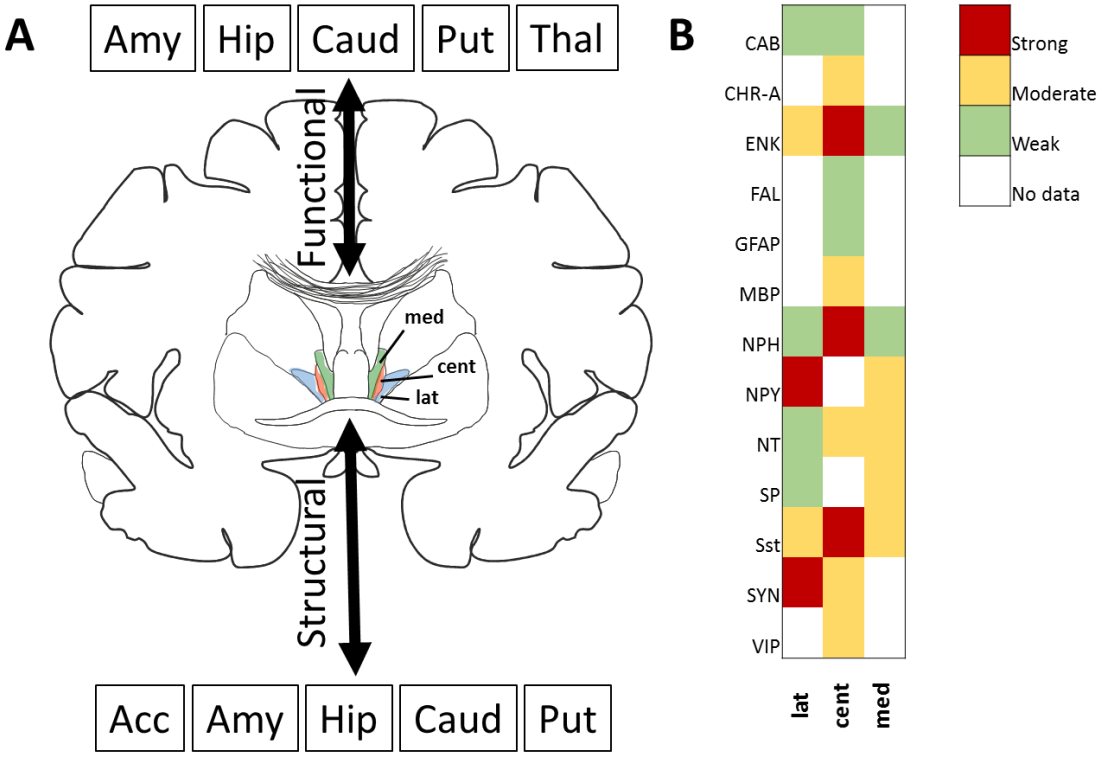


Figure 5. Anatomy, connectivity and chemoarchitecture of the human BNST. **A.** The human BNST is formed from three distinct subdivisions, medial, central, and lateral division (med, green; cent, red; lat, blue; respectively). Neuronal tracing studies, together with the use of fMRI technique, have revealed some of the functional and structural connections of human BNST, the most consistent findings of which are presented here. **B.** Heatmap of the expressions of different neuronal markers in the human BNST studied with immunohistochemistry, as described in Walter et al. 1991. *CAB – calbindin, CHR-A – chromogranin-A, ENK – enkephalin, FAL – fucosylacetyl lactosamine, GFAP – glial fibrillary acid, MBP – myelin basic protein, NPH – neurophysin, NPY – neuropeptide Y, NT – neurotensin, SP – substance P, Sst – somatostatin, SYN – synaptophysin, VIP – vasoactive intestinal peptide.*

spatial resolution. This means, e.g. in the case of the BNST, that the origins of the three pathway bundles are not known at the level of subdivisions. The recent functional MRI (fMRI) studies have revealed functional connectivity between the BNST and most of the regions structurally connected with it, including the amygdala, several regions of the basal ganglia, like the NAc, and putamen, as well as the hippocampus and thalamus (Avery et al. 2014).

The BNST is an intriguing brain region in that it has been, several times, shown to be sexually dimorphic (Allen and Gorski 1990; Zhou et al. 1995). The size of the BNST varies, being even as much as 2.5-times larger in males than in females. Giving this

finding social impact, Zhou et al. (1995) showed that the size differences are observable in transgender people, the BNST being smaller in female-identifying males than in male-identifying males, and vice versa. Avery et al. (2014) also showed sexually differing BNST connectivity between the male and female test subjects; for example, 76 % of the structurally connected brain regions showed greater connectivity in females than in male participants. While this finding seems initially intriguing and plausible, the authors note that the observed differences in the connectivity may rise from methodological limitations, and that further verification is needed.

From the translational research point of view, it is interesting to notice that the connections of the human BNST, both the structural and the functional, highly resemble the ones observed in rodents and non-human primates, implying at least some levels of evolutionary conservation of the structure across species (Dong and Swanson 2004a; Dong and Swanson 2004b; Oler et al. 2012). Similarly, as has been discussed, the human and rodent BNST share some histological characteristics, namely expressing largely the same neuropeptides (Moga et al. 1989; Walter et al. 1991). Despite the similarities, very little is, in fact, still known about the human BNST. For example, in humans, the BNST is proportionally larger than in rodents, indicating possible differences in the BNST circuitry (Lesur et al. 1989; Avery et al. 2014). Because of these points, and since the BNST is likely to become increasingly more interesting site as a drug target, more research in the human BNST is needed, in addition to more comparative approach in animal research concerning the BNST function.

3.3 Functional roles of the BNST

The BNST is often considered to be an integrative hub responsible for valence monitoring (Lebow and Chen 2016). In other words, the BNST receives sensory information from the cortex, as well as memory and emotion related inputs from the limbic system, and in the BNST these streams of information are integrated to produce either positive (rewarding, appetitive, pleasant) or negative (aversive) valence to inherently neutral external stimuli (Calhoon and Tye 2015). From the BNST the integrated information is sent back to the input areas in cortical and limbic systems as a feedback, but also to brain areas controlling physiological and homeostatic states, enabling proper response to

external environment or setting. Valence surveillance is therefore important in every form of behaviour, making decision on the reasonable action possible. The BNST – together with amygdala – is the main link in this neuronal circuitry. Therefore, disruptions in this link could cause disturbances in mood and stress processing, and reflecting this, the BNST has been implicated in some psychopathological states. The following will shortly review the known role of the BNST in both healthy stress and social behaviour, and in some behavioural disorders.

3.3.1 Stress

Biological stress is the physiological reaction and adaptation to external stressors, such as a real or an anticipated environmental or homeostatic challenge, or a threat to the well-being (Ulrich-Lai and Herman 2009; Stephens and Wand 2012). This adaptation is of major importance for the survival of an organism. The physiological changes are mediated by the central nervous system through the modulation of the HPA axis and the autonomic nervous system. These two systems are responsible for the changes in heart and respiratory rates, and in the blood pressure, to name a few. The BNST is indirectly connected to both of these systems through its output regions, especially the HPA axis-controlling hypothalamus, and the brainstem areas, like the PBN, and the nucleus of the solitary tract, responsible for the control of autonomic reactions (Magableh and Lundy 2014; Sosulina et al. 2006). Arguing for the role of the BNST, studies have shown that lesions in the anterior BNST decrease the HPA axis activation, while lesions in the posterior BNST contrarily increase it (Gray et al. 1993; Choi et al. 2007). Interestingly, also the size of the BNST has been shown to increase after chronic stress in rats, caused by the changes in dendritic arborisation (Vyas et al. 2003).

Some researchers link the BNST to stress-control solely because of its connections with the other brain regions known to be affected in stress: noradrenergic input from the *locus coeruleus* is often implicated in stress together with other adrenergic signalling, as are the raphe nuclei, also relevant in arousal, and the ventral subiculum, which is considered to inhibit the HPA axis (Andres et al. 1999; O'Mara 2005). While the exact role of the BNST in stress regulation is unknown, the different outputs and subregions are shown to act in separate parts of stress reactions, and even in opposing ways: Kim et al. (2013)

showed that optogenetic activation of oval BNST caused anxiogenic behaviours, whereas the activation of anterodorsal BNST (including roughly the anteromedial and the anterolateral BNST) was anxiolytic in nature. The same study also showed, that different outputs mediate different aspects of the behaviours, as the activation of the projections to lateral hypothalamus affected the open space avoidance (indicator of anxiety-like behaviour), but not the respiratory rate, while the opposite was true for the activation of the projections to the PBN. This shows that the BNST is not to be interpreted as one homogeneous brain region working as a filter, but as a multifunctional, and highly heterogeneous complex.

3.3.2 Social behaviour

Seeing that a negative social interaction can lead to physical threat, and a positive interaction could be an outset for reproduction, it is understandable that valence surveillance is tightly connected also to social encounters. Social behaviour network is known to consist of such nodes as the MeA, VTA, and hypothalamus (Newman 1999; Goodson and Kabelik 2009). The BNST is connected to this network again as a hub: parts of the BNST are known to connect to brain areas regulating aggression, defensive behaviour, mating and parental care, most important of which is the MeA, known to be important in both social recognition and social memory (Coria-Avila et al. 2014). Together these two brain regions, the BNST and MeA, have been shown to mediate partner preference and copulation behaviour, while aggressive behaviours are in part modulated by the vasopressin-androgen-projections from the BNST to the septal nuclei (Trainor et al. 2006; Coria-Avila et al. 2014). Data from Trainor et al (2006) indicates that estrogen-mediated activation of the BNST regulates the MeA activity, as they showed that c-Fos activation in the MeA after an aggressive encounter in mice positively correlated with estrogen receptor immunoreactivity in the BNST. The BNST is also connected with the habenula, a multifunctional set of nuclei, well known to be a mediator in sexual behaviours in rats (Rodgers and Law 1967; Modianos et al. 1975). Recently, a study showed that chronic social defeat, a model of psychosocial stress, increases ΔFosB expression in the BNST, indicator of repeated neuronal activation (Laine et al. 2017). The same study also showed increased BNST-activation, observed with MRI, in the stressed

mice compared to the controls, implicating the BNST in both, social behaviour and stress reaction.

From a more clinical perspective, changes in the BNST activation have been considered in human anti-social behaviours, like psychopathy (Lebow and Chen 2016). While both aggressive behaviours and impaired fear processing walk hand in hand with known roles of the BNST, no studies have actually been published that would link psychopathic tendencies with the BNST, other than indirectly by showing reduced amygdala activation (Marsh 2013). As will be discussed later in more detail, the BNST and amygdala do not always activate in synchrony, and therefore the role of the BNST cannot be extrapolated through the activation of the amygdala. As another example, human social anxiety is considered to be a disorder caused by dysfunctioning fear inhibition, social recognition, and motivation systems (Coria-Avila et al. 2014; Lebow and Chen 2016). In these behavioural systems, the BNST is seen again as an important information relay due its contacts with the frontal cortex, the fear-related amygdala, and the VTA, mediating the motivational aspects of the social encounters. Yet again, despite the hypothesised association, no studies showing BNST-related changes in patients diagnosed with any form of social anxiety disorders has been published.

3.3.3 Anxiety disorders

The rising interest in the BNST within the neuroscience community stems from the knowledge of the BNST activation during prolonged fear states (Lebow and Chen 2016). It has been known for decades – making it also an often-cited brain fact in non-scientific journalism – that the amygdala is activated during the moments of fear in humans and in other mammals (Spevack et al. 1975). Later, evidence for crucial role of a set of brain regions connected to the amygdala (hence named the “extended amygdala”) on the fear-related behaviours have been gathered (Davis et al. 2010). The BNST is a major part of the extended amygdala, and it is today known to be important in generating sustained fear states, or “anxiety,” distinct from phasic fear (referred here as “fear”) generated by the amygdala.

The difference between amygdala and the BNST has been shown by (Sullivan et al. 2004) in a test, where they lesioned the BNST and the CeA, and tested behavioural and physiological effects caused by different fear-conditioning paradigms. The lesions in the amygdala attenuated the freezing behaviour and the corticosterone responses in the cases of both the acute tone-conditioned and the context-conditioned fear stimuli. Meanwhile, the lesioning of the BNST did not have any effect on the responses to the tone, but disrupted the context-conditioned fear reaction. The same has been seen in a human study, where images of spiders were shown to patients with arachnophobia (Straube et al. 2007). The imagery alone caused activation in the BNST, as seen with fMRI, but not in the amygdala. In another, similar test, a live tarantula was brought to the same room with the non-phobic subjects: the closer the spider was brought to the person in the fMRI, the more activation in the BNST, but also in the amygdala, was observed (Mobbs et al. 2010). These data, together with evidence from many other studies, have been used to build a theory, where the BNST mediates the apprehension, and the anticipation of a distant threat, whereas both the BNST and the amygdala work together when the threat is immediate (Lebow and Chen 2013).

As described earlier, the amygdala and the BNST together form a circuit, through which the modulation of the stress state, via the HPA axis, is conveyed (Dong and Swanson 2004b). The idea is built upon the data showing CRF-expressing projections from the BNST to the amygdala activating the CRF-neurons in the CeA, which in turn activate the HPA axis (Davis et al. 2010). It is noteworthy, that BNST and amygdala are active parts of the homeostatic stress-circuitry, enabling the evolutionarily important “fight or flight” decision making and adaptation mechanisms with sequelae over generations.

Results implicating the BNST with anxiety are also increasingly relevant in clinical respect, as studies have shown differences in the BNST functioning in patients with generalized anxiety disorder, when compared to healthy controls (Yassa et al. 2012; Buff et al. 2017). Chronic treatment with fluoxetine, a selective serotonin reuptake inhibitor used in the treatment of anxiety disorders, has been shown to reduce stress-induced expression of immediate early gene c-Fos in the rat BNST, indicating decreased

activation (Bechtholt et al. 2007). Together, these data make BNST an interesting target region for future research in pharmaceutical treatments of anxiety.

Post-traumatic stress disorder, or PTSD, is a psychiatric condition characterized by sustained fear states following a traumatic event (Marshall and Garakani 2002). PTSD patients show changes in function of the HPA axis and the autonomous nervous system, and in cortisol levels, all of which are linked with the BNST. Animal models have implicated the BNST in PTSD-like chronic fear states, but no human study has shown any conclusive evidence on BNST dysfunction in PTSD patients (Etkin and Wager 2007; Bremner et al. 2008; Rodriguez-Sierra et al. 2016). Still, because of the known correlations between the BNST function and the observed PTSD dysfunctions, BNST-downregulation as a potential future pharmacotherapeutic strategy for PTSD is increasing in interest (Marshall and Garakani 2002; Fink 2011).

3.3.4 Addiction and withdrawal

Drug addiction is a chronic, relapsing disorder defined by compulsive drug seeking and concurrent relapse to drug use (Koob 2008; Koob and Volkow 2010). It is also characterized by activation of the brains stress systems, often considered to be activated as an opponent process to the excessive activation of the brains reward system, and to maintain the homeostasis in the chronic presence of the drug of abuse. Stress is also a well-known trigger for relapse. As discussed before, the BNST is an important relay in the formation of the stress state, and it has been shown that the stress increases CRF signalling in the BNST, which then induces drug-seeking behaviours in cocaine-trained rats (Erb and Stewart 1999; Partridge et al. 2016). The CeA is most likely also an important part of this circuitry, as it is one of the main sources of the CRF signalling in the BNST, and several drugs have been shown to affect the neurons in the CeA (Roberto et al. 2006; Chen et al. 2013). For example, Herman et al. (2013) showed that ethanol increases firing rates of CRF-neurons innervating the BNST.

The BNST is not linked to addiction only through stress, but is known to modulate the brain reward system as well (Georges and Aston-Jones 2001; Jennings et al. 2013). Kim et al. (2013) showed that optogenetic activation of projections from what they dubbed the

anterodorsal BNST (including anteromedial, anterodorsal, and juxtacapsular nuclei, but not oval nucleus) to the VTA induced a place preference, indicating positive valence. In another study, Jennings et al (2013) optogenetically activated the GABAergic and glutamatergic projections from the ventral BNST to the VTA. The activation of the glutamatergic projection was aversive in nature, whereas the activation of the GABAergic projection showed rewarding properties. Similarly, the inhibition of the BNST-VTA projections cancels the place preference induced by ethanol (Pina and Cunningham 2017). Rinker et al. (2017) linked the stress-induced CRF signalling in the BNST to the VTA by showing that the selective inhibition of the BNTS-VTA CRF-projections decreases binge like ethanol consumption in mice. The dopaminergic signalling works in the other direction as well, through the VTA projections to the anterior BNST: practically all of the drugs of abuse are known to increase dopamine signalling in the BNST, and the injection of dopamine receptor antagonist to the BNST is shown to decrease cocaine reinforcement (Epping-Jordan et al. 1998; Carboni et al. 2000; Meloni et al. 2006).

Two studies have shown, that the noradrenergic signalling to the BNST is critical in opiate withdrawal (Aston-Jones et al. 1999; Delfs et al. 2000). The BNST activation was shown to be increased during the opiate withdrawal, indicated by increased immediate early gene c-Fos activation, which was reduced by β-adrenergic receptor antagonist propranolol, directly administered to the BNST. Also, lesioning the noradrenergic projections from the caudal medulla to the BNST attenuated the opiate-withdrawal-induced place aversion.

The BNST has also been shown to go through adaptations and plastic changes in the presence of drugs of abuse (Korpi et al. 2015). For example, chronic administration of cocaine has been shown to increase the levels of plasticity related ΔFosB, and long-term administration of ethanol causes robust synaptic plasticity changes in the BNST (Weitlauf et al. 2004; Nunez et al. 2010).

Some data gathered has also implicated the BNST in human addiction (Avery et al. 2016). Two fMRI studies have been carried out, one showing increases in BNST activity in response to smoking cues in nicotine-addicted participants, while the other showed increased functional connectivity between the BNST and the amygdala in alcohol use

disorder patients relative to healthy controls (Dagher et al. 2009; O'Daly et al. 2012) While certainly more human studies on the BNST's role in addiction is needed, interest towards novel pharmacological interventions for addiction working through the BNST is rising (Avery et al. 2016). Some promising evidence has already emerged; a GABA superagonist gaboxadol shows aversive effects while activating neurons solely in the oval BNST, and is therefore hypothesized to be a potential anti-addictive drug (de Miguel et al. submitted). In summary, the BNST has been connected to several behavioural functions, some of which carry significant clinical implications (Table 3; Lebow and Chen 2016). From the clinical perspective, the data used as evidence have some caveats, like the small differences in structure and connectivity of the BNST between the humans and rodents that could, in the end, prove to be surprisingly significant, and the usage of the methodologically challenging fMRI in the human BNST studies, as discussed shortly before (Vul et al. 2009; Larsson et al. 2016). Still, despite these minor challenges, the growing evidence clearly suggest the BNST to be an important brain region in the control of behaviour, both in health and in several neuropsychiatric disorders.

Table 3. Some neuropsychiatric disorders in which the BNST has been implicated. DBS – deep brain stimulation; LTP – long-term potentiation; OCD – obsessive-compulsive disorder; PTSD – post-traumatic stress disorder.

Disorder	Implication of the BNST	Reference
Anxiety	Chronic stressors decrease BNST LTP	Conrad and Winder 2010
	Optogenetic activation of BNST modulates anxiety-responses	Kim et al. 2013
	Chronic psychosocial stress activates BNST	Laine et al. 2017
PTSD	Increased BNST activity in PTSD-like rats	Rodriguez-Sierra et al. 2016
OCD	DBS in BNST area alleviates symptoms	Nuttin et al. 1999; Islam et al. 2015
Phobias	Increased BNST activation and enhanced amygdala-BNST connectivity	Münsterkötter et al. 2015
Addiction	Increased BNST activity in response to smoking-cues in nicotine dependent	Dagher et al. 2009
	Bupropion, used in smoking cessation, increases catecholamine output in BNST	Cadeddu et al. 2014
	Chronic cocaine increases BNST plasticity markers	Nunez et al. 2010
Depression	Increased α-band oscillation in the BNST (relative to OCD patients)	Neumann et al. 2014
	Antidepressants increase catecholamine output in the BNST	Cadeddu et al. 2014
	BNST lesion blocks development of learned helplessness	Hammack et al. 2004
	BNST lesion enhances development of learned helplessness	Schulz and Canbeyli 2000

3.4 Somatostatin in the BNST

In the rodent BNST, Sst-expression is highly concentrated in the oval nucleus, and in slightly lesser extent in the juxtacapsular, the anteromedial and the anterolateral nuclei (Magableh and Lundy 2014; Nguyen et al. 2016). Otherwise, Sst-neurons are sparsely expressed in the BNST structure, with another smaller cluster of Sst-neurons in the posterior BNST (Moga et al. 1989). Sst-immunoreactive neurites can be seen in every BNST nuclei, which is considered to be an indication of Sst-innervation. This is also reflected by the observed expression of Sstr1, 2 and 4 in the BNST (Patel 1999). The study by Magableh and Lundy (2014) shows, by retrograde neuronal tracing, that Sst-neurons from the BNST innervate the PBN, together with CRF-projections from the same regions. In humans, as described earlier, Sst is largely concentrated in the central division of the BNST, where as many as 25 % of cell bodies are Sst-immunoreactive (Figure 5; Lesur et al. 1989; Walter et al. 1991). Sst-expression in the BNST can be considered intuitive because of the logical correlation between the proven anxiolytic properties of the Sst and the anxiety-related role of the BNST. Sst-signalling is known to be activated in response to stressors, and the activation of the Sstr-signalling has been shown to suppress CRF-induced stress responses(Shibasaki et al. 1988; Butler et al. 2012; Stengel and Tache 2017). Still, despite this evidence and the emergence of the modern genetic tools, like the transgenic animal models, optogenetics, and chemogenetics, no research on BNST Sst-neurons' roles in behaviour has been published.

Altogether, the BNST is known to be an important hub between the cortical and subcortical brain systems, mediating many stress- and affect-related responses (Calhoon and Tye 2015; Lebow and Chen 2016). Because of the vast expression of different receptors to target, and due to the rise of the neuron subtype-specific methods – like optogenetics and chemogenetics – to probe the characteristics of these receptor systems, the BNST is likely to become a target of pharmacological interventions in the future. The significance of the BNST in the control of homeostasis gives rise to some major difficulties in the drug development: for example, while the inhibition of the BNST might be able to reduce the symptoms in anxiety disorders, too broad inhibition of the site is very likely to cause severe HPA axis or autonomic nervous system related adverse effects. This is especially true because of the great abundance of CRF-neurons in the BNST, as it

is known that modulation of the CRF-system affects the HPA axis function (Ambrogio et al. 2008). Similar effects are also known to be present in clinically used antipsychotic and antidepressant drugs (Bhagwagar et al. 2002; Cohrs et al. 2006). In this regard, the knowledge that the BNST harbours a significant cluster of Sst-neurons could be used to facilitate the drug discovery (Walter et al. 1991; Bota et al. 2012; Nguyen et al. 2016). Since the Sst-signalling has been shown to counteract the effects of the CRF, the activation of the BNST Sst-neurons could be used as an anxiolytic or anti-addictive treatment: for example, it has been shown that CRF-antagonism in the VTA reduces anxiety caused by cocaine administration (Ettenberg et al. 2015). Similar effect could be possible through pharmacological activation of the BNST Sst-neurons, which would inhibit the BNST-VTA CRF-projections. To achieve this kind of pharmacotherapy, the nature of the BNST Sst-neurons should be known in more depth, and their potential role in modulating behaviour on their own should be addressed by further research.

4 AIMS OF THE STUDY

As reviewed, Sst is a widely expressed neuropeptide with implications in several brain functions, both in health and in disease. Importantly, Sst-system is considered to drive endogenous anxiolytic and stress-alleviating actions. Sst-neurons, on the other hand, are best known as interneurons in the cortex and hippocampus, but significant groups of Sst-neurons are also harboured in the amygdala and BNST. Both are known to be involved in the brain circuits mediating fear- and anxiety-related behaviours, stress reactions, and to be involved in addiction. Even though the existence of these Sst-neurons in the oval, anterolateral and juxtacapsular BNST has been known for years, there has been very little published research on them (Moga et al. 1989; Bota et al. 2012; Magableh and Lundy 2014). Anxiety disorders and addictions are a significant burden on society whereas the treatments of these disorders in still largely lacking. Finding a way to, for example, inhibit an overactive stress relay like the BNST in anxiety disorder patients would be a novel therapeutic strategy. To achieve this, more knowledge on the basic action of the neurocircuitry mediating the behaviours and reactions dysfunctioning in the neuropsychiatric disorders is needed.

In this work, I studied the innervation profile of the Sst-neurons in the anterodorsal part of the BNST (adBNST; constituting of oval, juxtacapsular, anterolateral, and anteromedial BNST) by viral anterograde tracing to show whether the Sst-neurons are local interneurons or projection neurons.

I also studied the role of the adBNST Sst-neurons in behaviours related to the BNST activation, particularly anxiety-, reward-, and drug withdrawal-related behaviours, with chemogenetics, utilising a set of designer receptors exclusively activated by designer drugs (DREADDs; Zhu and Roth 2015).

5 METHODS AND MATERIALS

5.1 Experimental animals

For the behavioural studies, a mouse line expressing Cre-recombinase enzyme in Sst-neurons ($Sst^{tm2.1(cre)Zjh}$/J; Jackson Laboratories) was used. The tracing studies were done using a transgenic mouse line, acquired by breeding the Sst-cre mice with a reporter mouse line (B6.Cg-Gt(ROSA)26$Sor^{tm14(CAG-tdTomato)Hze}$/J; Jackson Laboratories) resulting in a mouse line with tdTomato fluorescent reporter protein expressed together with Cre-recombinase in Sst-neurons. Male mice aged between 6 – 16 weeks, weighing 20 – 35 g at the beginning of the experiments, were group housed (2 – 4 mice per cage) in individually ventilated cages with 12-hour light cycle (lights on at 06.00 am), standard aspen bedding, nesting material and a plastic in-cage housing, with water and rodent chow available *ad libitum*.

Originally, I tested both males with homozygote and heterozygote genotype on the somatostatin-cre mouse line for behavioural changes, but excluded the homozygote mice from the analysis as per emerged update from the animal provider, due to findings of abnormal Sst-RNA expression and behavioural phenotypes (unpublished data, Jackson Laboratories). All the animal tests were approved by the Provincial Government (permission ESAVI/3806/04.10.07/2015) and conducted in accordance with the national and university-level ethical and procedural guidelines.

5.2 Virus constructs

For the neural tracing study AAV2/8-Cag-Flex-Myr-eGFP ($4x10^{12}$ genome copies/ml) construct was used, purchased from Neurophotonics Center (CERVO Brain Research Centre, Quebec, Canada). The viral vectors used in the DREADD-driven behavioural studies were built upon the following constructs: rAAV8/hSyn-DIO-hM3Dq-mCherry ($5,9x10^{12}$ genome copies/ml) for the transfection of the Gq-coupled designer receptors, and rAAV8-hSyn-DIO-mCherry ($6,4x10^{12}$ genome copies/ml) for the mCherry expressing controls, both obtained from University of North Carolina Vector Core.

5.3 Animal surgery and stereotaxic viral injections

The mice were anesthetized with a mixture of isoflurane (4 % for induction, 0.5 – 2 % for maintenance) and oxygen (flow rate 0.8 – 1 l/min), after which they were placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Before the opening incision on the scalp, iodopovidone was applied on the surgical region, and lubricative eye gel applied to prevent corneal damage. Small craniotomies were made above the target regions and viruses were injected with a self-made steel cannula (30 Gauge) connected to a Microliter syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a pump system (Univentor Ltd, Zeijtun, Malta). Stereotaxic coordinates, relative to bregma, AP +0.2 mm, ML ±1.0 mm DV -4.0 mm were used for BNST, chosen based on the the Mouse Brain Atlas (Franklin and Paxinos 2008) and verified with dye injections. For the tracing studies, a unilateral injection of 120 nl was made, whereas for the DREADD-delivery two, bilateral injections of 120 nl were made (Figure 6A). After the injection, the injector was first held at the injection spot for 5 minutes and then slowly withdrawn to avoid upward flow of the liquid. The mice were sutured with Ethicon Perma-Hand silk or PDS*II polydioxanon monofilament sutures. They were administered 1 mg/kg carprofen (s.c.) for postoperative analgesia, and were let to recover from anaesthesia in a 37 °C incubator until ambulatory. All the tests, behavioural and immunohistochemical, started 2 weeks after the last viral injections to allow adequate receptor expression.

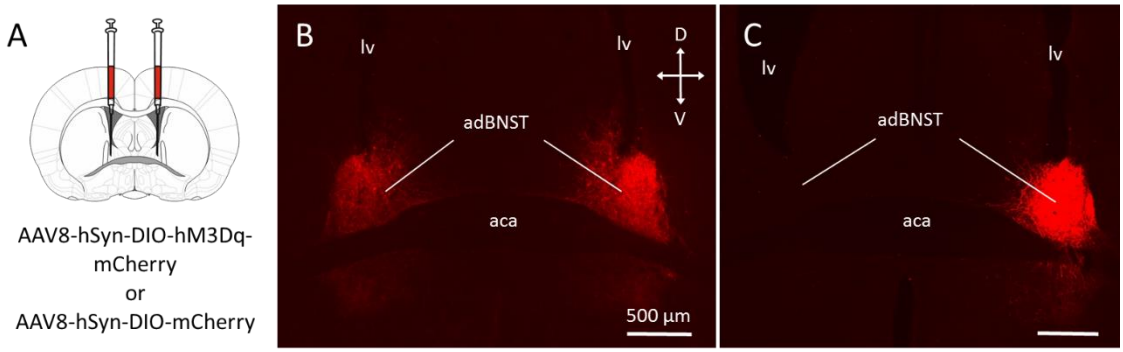


Figure 6. Sst-neuron targeting of adBNST neurons. **A**. A schematic of the bilateral stereotaxic delivery of vectors encoding activating Gq-coupled designer receptor or mCherry protein as a control. **B - C.** Representative sections showing the case examples of successful bilateral transfection (**B**), and a unilateral transfection (**C**). *aca – anterior commissure; adBNST – anterodorsal part of the BNST; lv – lateral ventricle.* The scale bars indicate 500 µm.

5.4 Behavioural studies

The mice were habituated to handling for two days prior to the first behavioural assessment. All tests were carried out in the morning time between 7.00 AM – 11.00 AM unless otherwise stated. For each behavioural test, the mice were brought to the adjacent room to habituate at least 1 h before the first test trial, and at least 30 min before clozapine-N-oxide (CNO) pre-treatment (1 mg/kg, i.p.). The behaviour was recorded and analysed using the EthoVision software (Noldus Information Technologies, Leesburg, VA, USA).

5.4.1 Elevated plus-maze

For the assessment of anxiety-like behaviour the elevated plus-maze test was performed. The mice were placed in the centre of the maze (40 cm long arms, 40 cm above the ground), facing always the same closed arm, 30 min after the CNO injection and allowed to freely explore the maze for 5 min. Test was performed at the light intensity of 175 lx. The time spent in the open arms, head dips over the edge of the open arms, and exploratory stretching behaviour were measured. Open arm entries were interpreted as a behaviour where the mouse has entered the arm with all the four paws, whereas the partial entries to the open arms, in which at least one of the paws remains in the centre area or in the closed arm, were interpreted as stretch-attend postures. Head dips and stretching behaviour were manually scored from the recorded video files.

5.4.2 Open field and novel object exploration

To further evaluate anxiety and exploratory activity, a two-phased open field test was performed. In the first phase, the mice were placed in the centre of an open arena field (50 x 50 cm) and were allowed to freely explore the arena for 3 min. A round plastic object (diameter 4 cm) was quickly placed in the centre of the arena and the mouse behaviour was then recorded for another 3 min (Vekovischeva et al. 2013). Testing was performed at the light intensity of 175 lx. For the analysis of the behaviour, the arena was divided into central and peripheral areas so that the 25 % of the total area in the centre dedicated as the centre. Time spent in the centre of the arena and the total distance moved were assessed through the whole 6 min trial, as well as separately for the first and the

second 3 min phase. Object related exploration was manually scored from the video record as contacts with the novel object.

5.4.3 Conditioned place preference

A biased place conditioning paradigm was used to assess the possible rewarding or aversive effect of the CNO-induced activation of BNST. The paradigm was built of three phases: in the first, the pre-conditioning phase, the mice were, for 15 min, presented with two distinct floor materials, a metal plate with 5 mm holes 3 mm apart, and a plastic grid with 2 cm wide bars 0.5 cm apart, placed in 1:1 relation covering the floor of each Plexiglass cage (19 x 36 cm). The pre-test was repeated three times and the time spent on the preferred floor material on the third test was used as a basis of the behavioural analysis. During the conditioning phase, the cage floor was covered solely with either of the two materials at the time. The daily routine consisted of two 30 min trials, one in the morning (between 7.00 – 11.00 AM) and one in the evening (between 4.00 – 7.00 PM). In the morning trial, the mice were injected with 10 ml/kg 0.9 % saline (i.p) 30 min prior to placing them on the preferred floor material. In the evening trial, the mice were injected with 1 mg/kg CNO (i.p.) 30 min prior to placing them in the cage with the non-preferred floor material. The daily conditioning routine was performed on four consecutive days. The post-conditioning assessment of the aversion was performed 48 h after the last conditioning trial. The mice were again placed in cages with 1:1 ratio of the two floor materials and the time spent on each material was assessed. The trial was repeated in a similar fashion the next day, but with 10 ml/kg 0.9 % saline injection 30 min prior the test. The difference in the time spent on the non-preferred floor material during the third pre-conditioning and the post-conditioning tests were used as the measure of reward or aversion (i.e. time-shift).

5.4.4 Naltrexone-precipitated morphine withdrawal

The mice were injected 10 ml/kg volume morphine (s.c.) twice daily, in the morning between 8 – 10 AM, and in the afternoon, between 5 – 6 PM, with the dose progressively increasing from 8 to 45 mg/kg over a period of 5 days (Figure 11A; Suzuki et al. 1996; Vashchinkina et al. 2017). In the fifth day, 2 h after the final morphine administration, the mice were injected with 1 mg/kg CNO (i.p.), and 30 min after the CNO the withdrawal

symptoms were precipitated by injecting 3 mg/kg naltrexone (s.c.). The mice were immediately after the naltrexone administration placed in transparent acrylic cylinders (30 cm height, 20 cm diameter), and the behaviour was recorded with a video recorder (Sony HDR-CX) for the following 30 min. The number of jumps, exploratory rearings, and forepaw tremors were manually scored with EthoGraph 2.06 software (Ritec, St. Petersburg, Russia).

5.5 Immunohistochemistry and imaging

To enhance the fluorescence signal for the neural tracing study, immunostaining was performed for the GFP protein. All the mice were anesthetized with pentobarbital (100 mg/kg i.p.) and transcardialy perfused with first 1x PBS followed by 4 % PFA. The dissected brains were fixed in 4 % PFA at 4 °C for 12 hours, then transferred to 30 % sucrose in PBS until the brains were completely submerged, at least for 48 h. The brains were then frozen with isopentane and stored in -80 °C until sectioned. 80 µm coronal sections were cut throughout the brain with cryostat (CM3050S, Leica Biosystems, Wetzlar, Germany). For immunostaining, the sections were washed at room temperature in 1x PBS (5 min, 3 times), blocked with 1 % BSA with 0,3 % Triton X-100 in 1x PBS for 1 h at room temperature. The sections were then incubated with the primary antibody **(**chicken anti-GFP**)** overnight at 4 °C, washed with 1x PBS (5 min, 3 times) and incubated with secondary antibody (goat anti-chicken with Alexa Fluor 488**)** for 2 h at room temperature. Sections were then washed once more with 1x PBS (5 min, 3 times), mounted on slides, and coverslips were applied with FluoroSave mounting medium. Mounted sections were imaged with AxioImager epifluorescent microscope (20x Plan Apochromat dry immersion objective, numerical aperture NA 0.8; 40X EC Plan Neofluar dry immersion objective, NA 0.75; 100x EC Plan Neofluar oil immersion objective, NA 1.3) and Axio Scan.Z1 slidescanner (20x Plan Apochromat dry immersion objective, NA 0.8; both by Carl Zeiss AG, Oberhocken, Germany) for double-labelled fluorescent neurites as a marker for projections.

To verify the correct localization of the DREADD-carrying vector injections, immunostaining was performed for the mCherry fluorescent protein using the same protocol described above, except for collecting 40 µm coronal sections and using primary

rabbit anti-mCherry and secondary goat anti-rabbit with Alexa Fluor 594 antibodies. The mounted sections were imaged with AxioImager epifluorescent microscope, and analysed to identify the locations of the transduced neurons. Only the animals with successful viral transduction localized at adBNST area were included in final data analysis.

5.6 Drugs

The clozapine-N-oxide (CNO; Carbosynth Ltd., Berkshire, UK) was dissolved in dimethyl sulphoxide (DMSO; 0.5 % of total volume) and diluted with 0.9 % saline. Morphine hydrochloride and naltrexone (both from Yliopiston Apteekki, Helsinki, Finland) were initially dissolved in, and diluted with 0.9 % saline. Carprofen (Norocarp vet 50mg/ml, Norbrook, Newry, UK) and pentobarbital (Mebunat vet 60 mg/ml, Orion Pharma OYj, Espoo, Finland) were diluted with 0.9 % saline. Isoflurane (Vetflurane 1000 mg/g, Virbac S.A., Carros, France) was vaporized and mixed with oxygen with VetEquip (Livermore, CA, USA) isoflurane vaporizer.

5.7 Antibodies

For the DREADD verification immunohistochemistry, rabbit anti-mCherry (ab167435, Abcam, Cambridge, UK) was used as a primary antibody, diluted 1:800 in the blocking solution. As a secondary antibody, donkey anti-rabbit Alexa Fluor 594 (ab150076, Abcam) was used, diluted 1:1000 in the blocking solution. For the tracing fluorescent enhancement immunohistochemistry, chicken anti-GFP (ab13970, Abcam) was used as a primary antibody, and goat anti-chicken Alexa Fluor 488 (ab150169, Abcam) as a secondary antibody, both diluted 1:800 in the blocking solution.

5.8 Reagents and solutions

The solutions and the reagents, apart from the drugs and antibodies, used in the study are presented below in the Table 4.

Table 4. The reagents and solutions used in the study.

Reagent	Vendor
10 x phosphate-buffered saline (PBS)	
1.3 M NaCl	Fluka
70 mM Na_2HPO_4	Fluka
30 mM NaH_2PO_4	Fluka
dH2O	
4 % Paraformaldehyde (PFA)	
Paraformaldehyde, 96 %	Acros Organics
1 x PBS	University of Helsinki
30 % Sucrose solution	University of Helsinki
Isopentane	Fisher BioReagents
Antifreeze solution	
25 % Glycerol	Fisher BioReagents
25 % Ethylene glycol	Fisher BioReagents
1 x PBS	University of Helsinki
Blocking solution	
1% Bovine Serum Albumin	Sigma Aldrich
0.3 % Triton X-100	BDH Laboratory Supplies
1 x PBS	University of Helsinki
FluoroSave Reagent	Millipore
Dimethyl sulphoxide (DMSO)	Sigma Aldrich

5.9 Data analysis

In the tracing and DREADD experiments, the verification of the fluorescence signal location was qualitatively analysed *post hoc* with Zen software (Carl Zeiss AG, Oberhocken, Germany). Following the verification of the DREADD-expression, the mice were grouped into bilateral (Figure 6B, n=8), and unilateral (Figure 6C, n=10) hM_3D_q-DREADD groups (later referred to as bilateral and unilateral group, respectively), whereas the mice with no visible DREADD expression despite the injection (n=3) were added to the control group (total n=23). Statistical analysis was conducted using SPSS software version 24 (IBM Analytics, Armonk, NY, USA), and all the graphs were generated by using Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Shapiro-Wilk's test was used to test for the normality of the data. For the analysis of the behavioural data, the comparisons across more than two groups were made by using a standard one-way analysis of variance (ANOVA). Differences between the populations were considered to be significant at P values below 0.05. All the data described and shown are presented as mean ± 95 % CI, unless otherwise stated.

6 RESULTS

6.1 Anterograde tracing of the adBNST Sst-neuron outputs

To study the efferents of the adBNST Sst-neurons, I unilaterally expressed Cre-inducible green fluorescent protein (GFP) in the adBNST of Sst-Cre-tdTomato reporter line mice (n=2). The adBNST showed fluorescent neurites (Figure 7A) and double-labelled neuron somas (Figure 7B) in the injection site, and robust local innervation all around the BNST. Interestingly, neurites visibly expressing both the native tdTomato-fluorescence, and the GFP-fluorescence from the injection were only visible near the injection site (not shown), but not further away in the proclaimed projection sites.

A brainwide screening for GFP-labelled neurites revealed a robust innervation in the MeA (Figure 7D), and in slightly lesser extent in the CeA, to which the projections followed the *stria terminalis* (Figure 8A), and through the so called ventral amygdaloid pathway, or *ansa peduncularis*, proceeding through the lateral hypothalamus (LH), near the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), and the *substantia innominata* (Larriva-Sahd 2004). Interestingly, there were no visible fluorescent neurites in the basolateral amygdala (BLA; Figure 7D), and only very few in the highly Sst enriched area of the medial part of the CeA (Figures 7D and 8A). Fluorescent neurites were also observable in higher abundance in the lateral hypothalamus (LH; Figures 7D and 8B). Anterior to the BNST, fluorescent neurites were seen to proceed towards NAc and ventral pallidum (VP; Figure 7C), but only a few fluorescent neurites were visible in NAc more anterior, at the level of frontal cortex. No innervation was visible in the VTA, or in the *substantia nigra*, but fluorescent neurites were observable in the lateral PAG (not shown), and in the PBN (Figure 7E). The tracing revealed that the projections are ipsilateral, as no neurites were seen on the contralateral hemisphere. No identifiable endpoints for the projections, like perisomatic baskets, were observable (Dabrowska et al. 2016). All the findings described were consistently observed in every screened mouse. Taken together, the adBNST Sst-neurons innervate both the local BNST network, and other brain areas (Figure 8C).

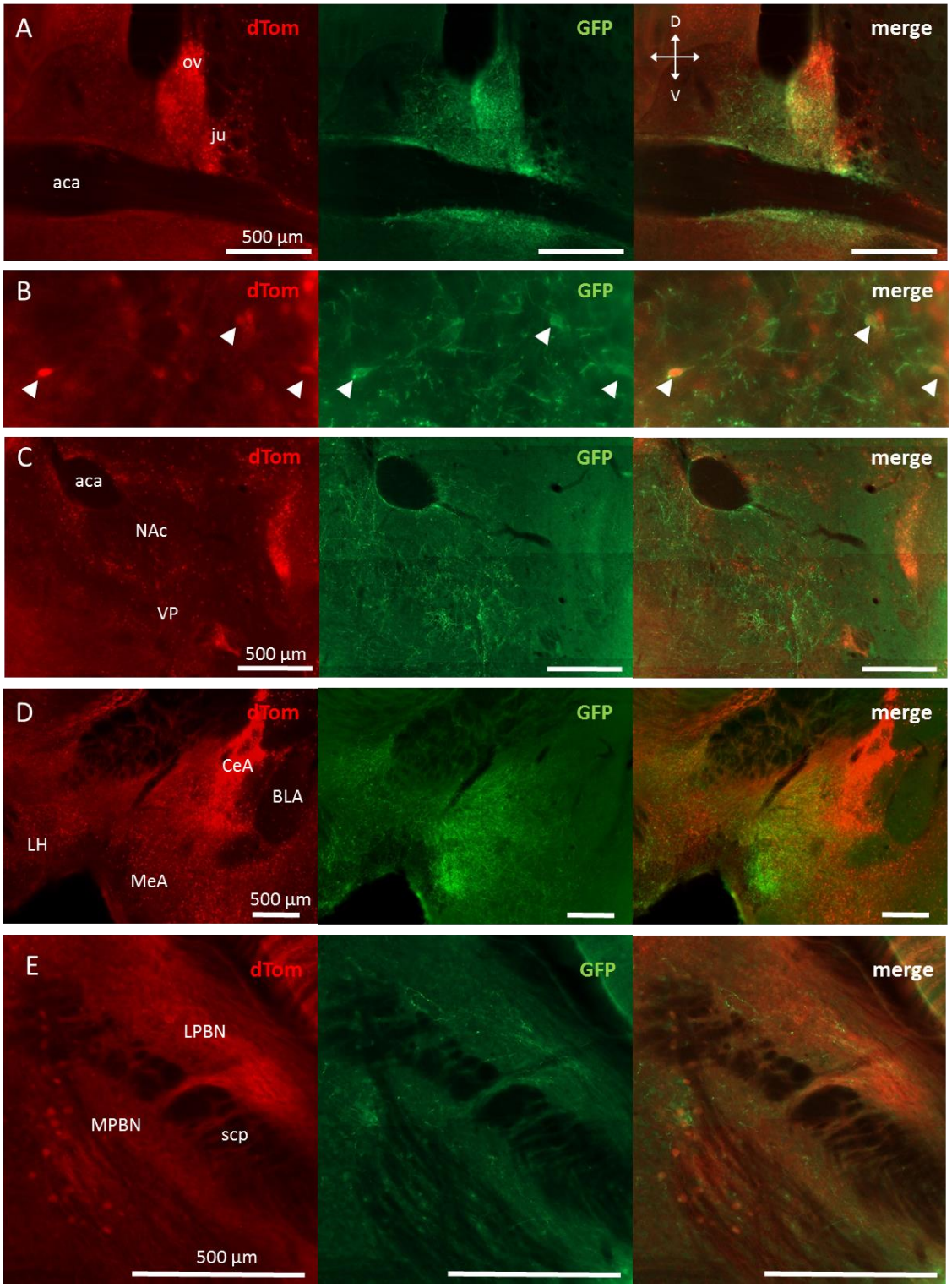


Figure 7. Anterograde neuronal tracer injected into adBNST reveals Sst-neurons projecting to stress-mediating brain regions. **A.** Green fluorescent neurons on the injection site are most prominent in the subnuclei rich in Sst, oval (ov) and juxtacapsular (ju) nuclei, but also visible in the anteromedial and anterolateral nuclei. **B.** In the BNST, neuron somas expressing GFP-fluorescence from the tracer as well as the tdTomato-fluorescence of the Sst-neurons of the reporter mouse line. **C.** Anterior to the injection site, GFP-fluorescent neurites are observable in moderate density in the ventral pallidum (VP) and in the *nucleus accumbens* (NAc). **D.** The most abundant expression of GFP-fluorescent neurites is observed in the medial amygdala (MeA), the medial part of the central nucleus of the amygdala (CeA), and in the lateral hypothalamus (LH). Neurites are virtually not present in the basolateral amygdala (BLA). **E.** The tracing also strengthened the data from previous research showing BNST Sst-neurons projecting to parabrachial nucleus (PBN), as fluorescent neurites were visible both in the medial (MPBN) and the lateral PBN (LPBN). dTom – native tdTomato-fluorescence from the Sst-neurons of the reporter mouse; GFP – GFP-fluorescence from the injected tracer; aca – anterior commissure; scp – superior cerebellar penduncule. The scale bar denoted 500 µm.

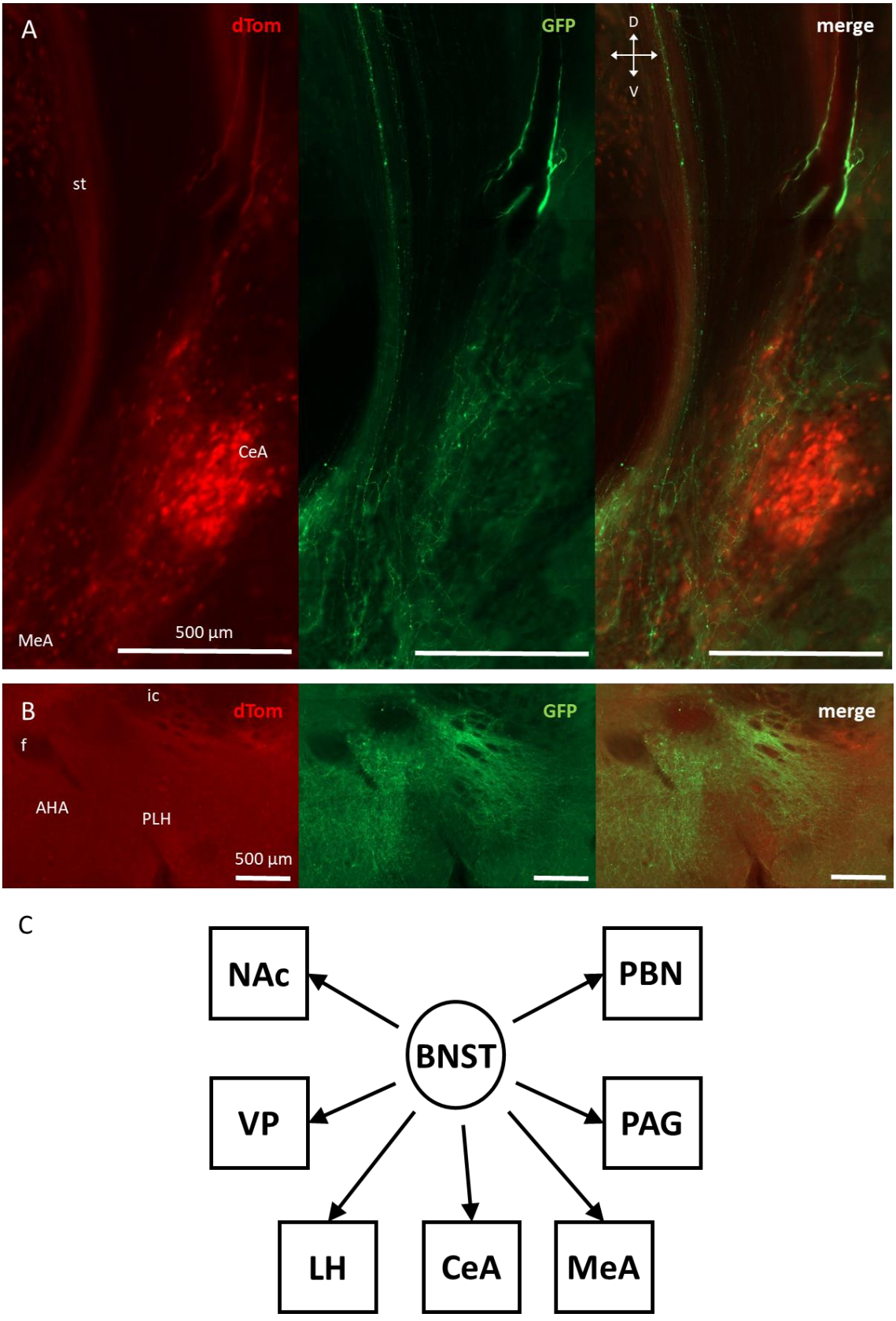


Figure 8. The adBNST Sst-projections follow known BNST output bundles. **A.** A set of GFP-fluorescent neurites followed the *stria terminalis* (st) to the amygdala. **B.** GFP-fluorescent neurites were densely observable in the hypothalamic areas (here anterior hypothalamic nucleus (AHA) and posterior lateral hypothalamus (PLH), and, in part, ending up to the amygdala, very closely following the ventral amygdaloid pathway, or the *ansa peduncularis*, going around the internal capsule. **C.** Schematic representation of the major observed output areas of the adBNST Sst-neurons. NAc – *nucleus accumbens* core; VP - ventral pallidum; LH – lateral hypothalamus; MeA – medial amygdala; PAG – periaqueductal gray; PBN – parabrachial nucleus. dTom – native tdTomato-fluorescence from the Sst-neurons of the reporter mouse; GFP – GFP-fluorescence from the injected tracer. The scale bar denotes 500 µm.

6.2 Anxiety-like behaviour after specific activation of the adBNST Sst-neurons

To study how the selective activation of the adBNST Sst-neurons affects acute anxiety-like behaviour, the mice were pre-treated with CNO (1.0 mg/kg i.p.) and, 30 min later, were tested with elevated-plus maze and open field paradigms (Figure 9A). In the elevated-plus maze, the CNO-induced activation of the Sst-neurons did not have any effect on the movement of the mice in the different groups (Figure 9B; $F(2,38)=0.364$, $p=0.139$, 95%CI [17.13 – 23.22]). At the same time, it did not seem to have any significant effect on the time the mice spent in the open arms (Figure 9C; $F(2,38)=1.15$, $p=0.327$ 95%CI [43.39 – 62.49]), nor in the exploratory activities, like the frequency of the stretch-attend postures (Figure 9D; $F(2,38)=0.276$, $p=0.191$, 95%CI [10.28 – 13.48]) or the head dips (Figure 9E; $F(2,38)=1.930$, $p=0.159$, 95%CI [13.43 – 21.88]).

In line with the above results, no effect by CNO on the movement between the groups was observed in the open field test (Figure 9F; $F(2,38)=1.782$, $p=0.183$, 95%CI [34.53 – 38.81]). Similarly, no differences in anxiety-related behaviours, like in the time spent in the centre of the arena (Figure 9G-I; total $F(2,38)=0.658$, $p=0.534$, 95%CI [45.74 – 59.70]; first 3 min $F(2,38)=0.142$, $p=0.524$, 95%CI [22.05 – 28.89]; last 3 min $F(2,38)=1.53$, $p=0.229$, 95%CI [20.24 – 28.31]) or in the number of contacts with the novel object during the last phase of the test (Figure 9J; $F(2,38)=0.339$, $p=0.715$, 95%CI [0.75 – 6.42]), were observed. Altogether, the two used behavioural paradigms failed to demonstrate anxiety-related effects of the CNO-induced activation of the adBNST Sst-neurons.

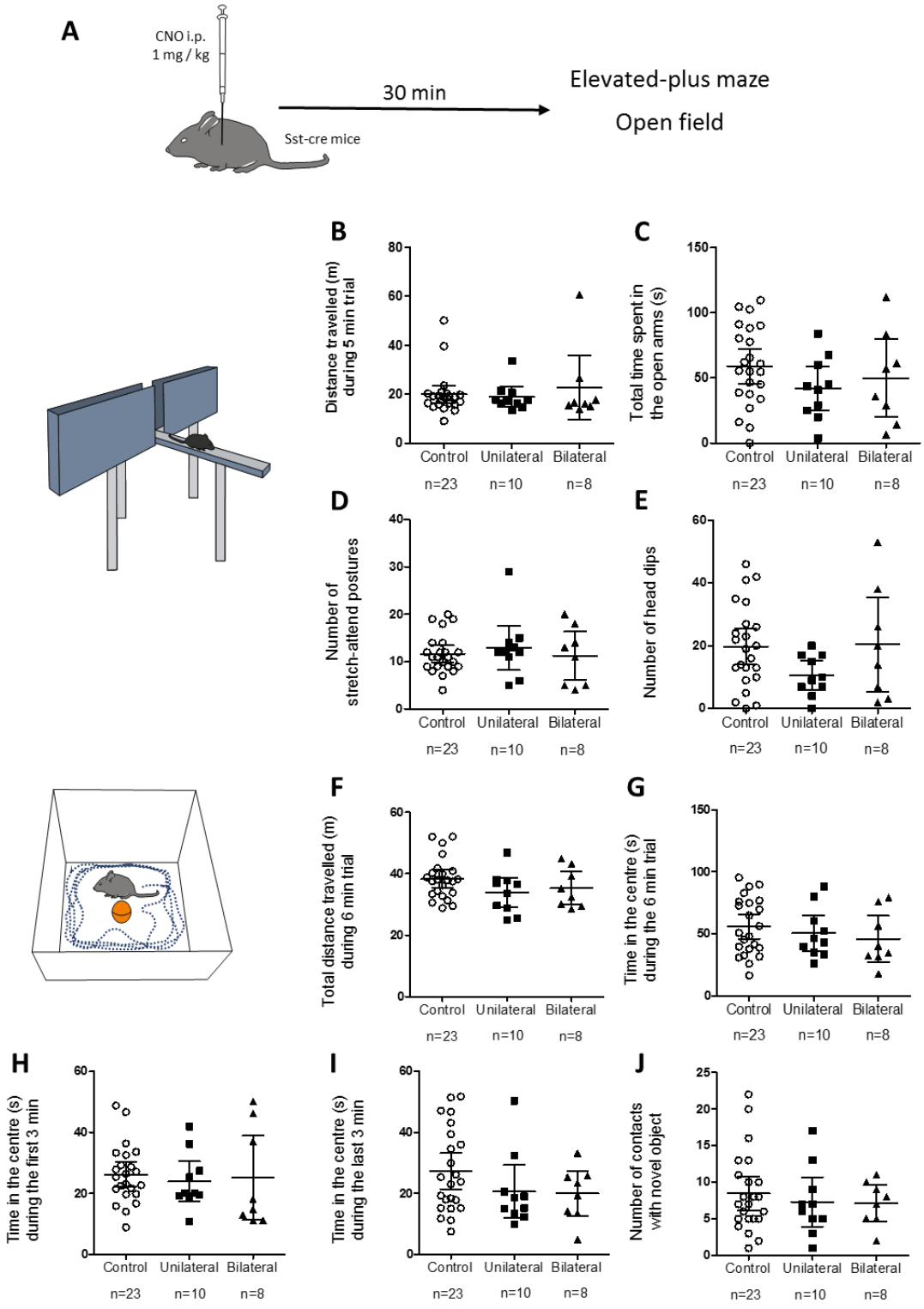


Figure 9. CNO-induced activation of adBNST Sst-neurons did not show effects on anxiety-like behaviour. **A**. The Gq-mediated activation of the Sst-neurons was induced with 1 mg/kg CNO i.p. 30 min prior the behavioural testing. Elevated-plus maze did not show statistically significant differences between the groups in movement (**B**), or in time spent in open arms of the maze (**C**), nor in exploratory behaviours, stretches (**D**) or head dips (**E**). Open field with novel object exploration showed no statistically significant differences in movement (**F**), nor in anxiety-related parameters like time in spent in the centre of the arena in different time frames (**G-I.**) or the number of contacts made with the novel object (**J**). All data presented as mean ± 95 % CI.

6.3 Reward-related behaviours after specific activation of the adBNST Sst-neurons

I used a biased conditioned place preference test (CPP) to study the potential role of the adBNST Sst-neurons in rewarding behaviours. Locomotor activities during the conditioning trials were similar between the groups, with an exception in form of an observable difference during the 4th vehicle conditioning trial between the bilateral and control groups (Figure 10A, vehicle-DREADD $F(2, 114)=4.15$, $p=0.0234$) CNO-DREADD $F(2,114)=2.19$, $p=0.1257$). The CPP test failed to show any statistically significant rewarding or aversive properties of CNO-induced activation of the adBNST Sst-neurons (Figures 10B and C, time-shift $F(2,38)=0,580$, $p=0,565$ 95%CI [-22.953 – 39.123]; time-shift with saline injection $F(2,38)=1,080$, $p=0.350$, 95%CI [-19.516 – 68.269]). Taken together, the CNO-induced activation of the adBNST Sst-neurons did not impact place conditioning.

6.4 Withdrawal symptoms after specific activation of the adBNST Sst-neurons

To elicit the adBNST Sst-neurons' role in giving rise to withdrawal symptoms, I used a method to precipitate the withdrawal symptoms with naltrexone in subchronically morphine-treated mice (Suzuki et al. 1996; Vandergriff and Rasmussen 1999). All the mice showed the predetermined symptoms, but the test failed to show any differences between the study groups in the number of jumps (Figure 11B, $F(2,14)=1.158$, $p=0.343$, 95%CI [27.41 – 44.71]), rearings (Figure 11C, $F(2,14)=0.009$, $p=0.991$, 95%CI [25.12 – 41.23]), or front paw tremors (Figure 11D, $F(2,14)=0.09$, $p=0.914$, 95%CI [142.56 – 216.74]), nor in the total duration of the tremors (Figures 11E and $F(2,14)=0.495$, $p=0.620$, 95%CI [24.92 – 27.22]). Altogether, the test here failed to demonstrate any morphine withdrawal-related effects of the CNO-induced activation of the adBNST Sst-neurons.

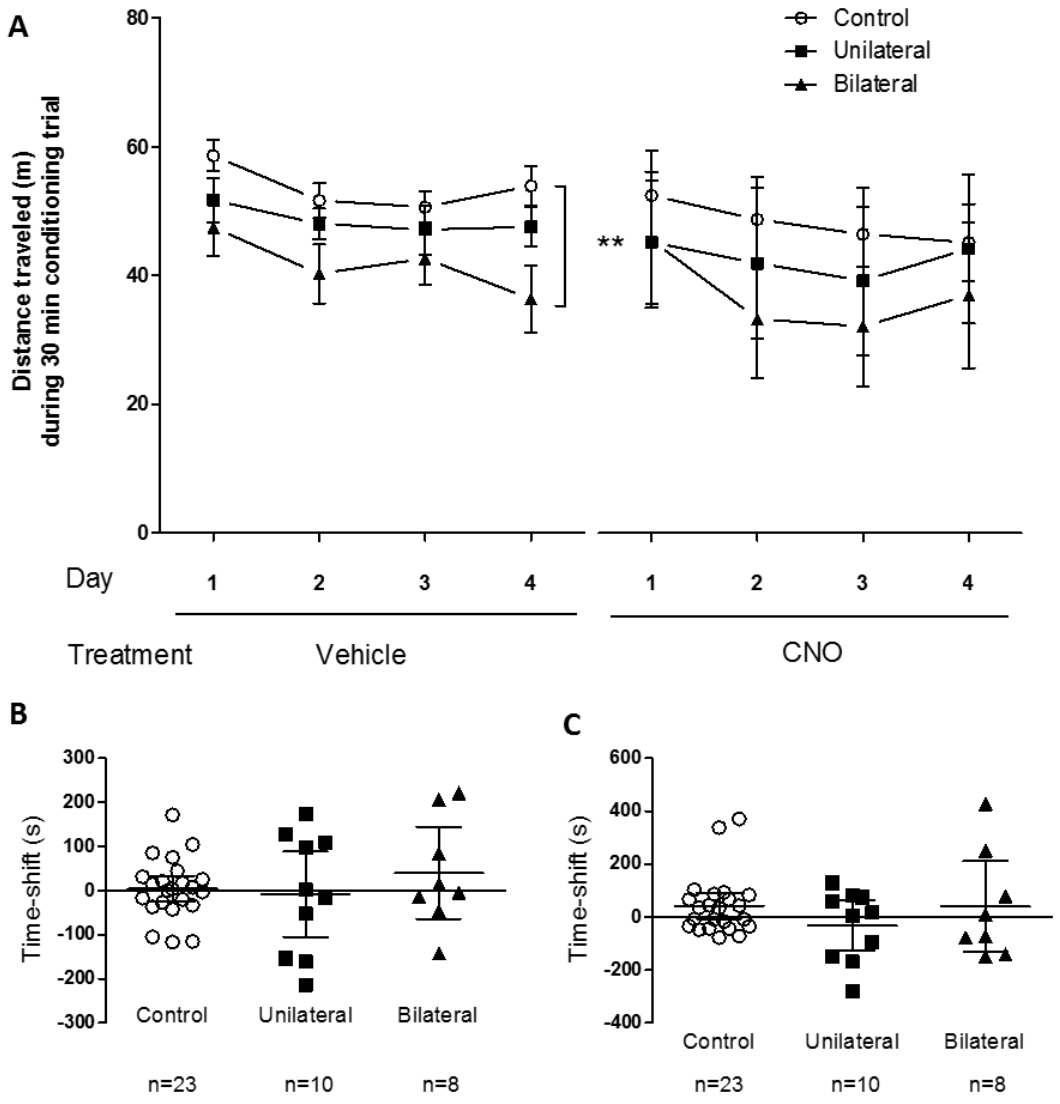


Figure 10. CNO-induced activation of the adBNST Sst-neurons showed no place conditioning effects. **A**. Changes in the locomotor activity between the groups during the conditioning were observed with a significant difference on the day 4 vehicle conditioning between the control and the bilateral group. **B-C**. Time-shift was measured as the difference in time the mice spent on the non-preferred floor material on the post-conditioning tests and the third pre-conditioning test. The test failed to show any significant differences between the groups without (**B**) or with a 0.9 % saline injection 30 min prior to the testing (**C**). All data presented as mean ± 95 % CI.

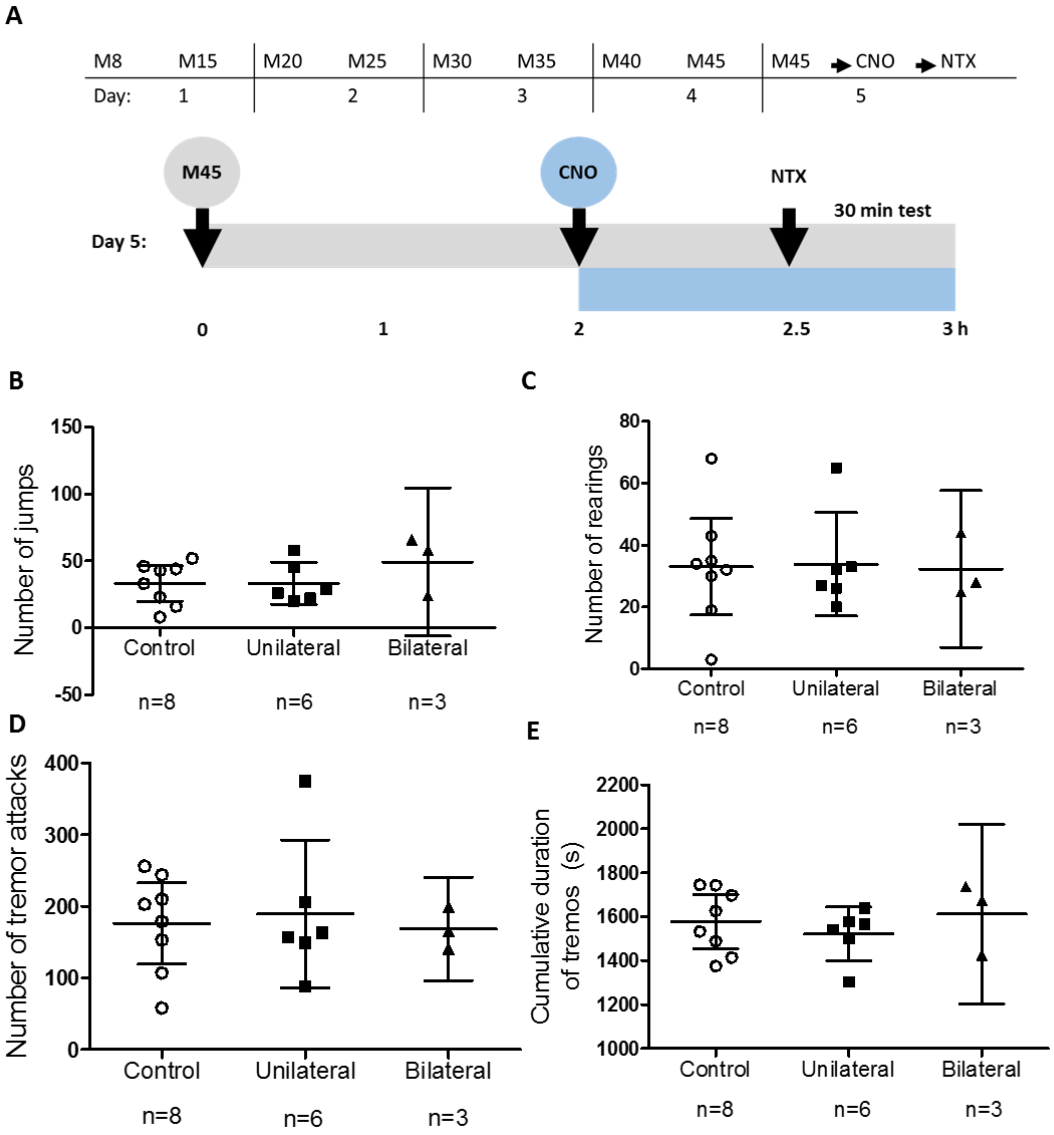


Figure 11. CNO-induced activation of adBNST Sst-neurons failed to show effects on morphine withdrawal symptoms precipitated by naltrexone. **A.** The mice were subchronicaly treated with increasing doses of morphine (8 to 45 mg/kg s.c. twice a day) during 4 consecutive days. On the day 5 the mice received 45 mg/kg morphine s.c,.followed by 1 mg/kg CNO-induced activation of the Sst-neurons, and 30 min after that 3 mg/kg naltrexone to precipitate the withdrawal symptoms. **B-E**. There were no significant differences in withdrawal symptoms scored during the 30 min following naltrexone administration. All data presented as mean ± 95 % CI.

7 DISCUSSION

Neuropsychiatric disorders cause enormous social harm and financial loss worldwide (DiLuca and Olesen 2014). Still, only very few new therapeutics have emerged during the past years, and one of the main reason causing this is our lacking knowledge about the aetiology the disorders (Nutt and Goodwin 2011; Kesselheim et al. 2015). In the light of this problem, research on neuronal circuits behind the disrupted behaviours is an important approach towards the goal of finding new potential drug targets and therapeutic strategies (Song and Knöpfel 2015; Hempel et al. 2017). The BNST has been shown to be an important part in the brain circuit active in anxiety and addiction, whereas the endogenous Sst-system has been shown to work in anxiolytic fashion (Lebow and Chen 2016; Stengel and Tache 2017). Therefore, learning how the Sst-neuron population works in the BNST could prove to be very beneficial, for example, if it would be possible to activate the Sst-system to inhibit the overactive BNST to alleviate anxiety- and addiction-related symptoms. To this end, the aim of this study was to characterise the Sst-neuron population in the anterodorsal parts of the BNST, and to probe their role in the neuropsychiatrycally relevant behavioural models.

7.1 The adBNST Sst-neuron projections

First, to demonstrate, whether the Sst-neurons are projecting neurons, I used a cell-type specific neuronal tracing technique with Sst-neuron reporter mouse line. I was able to demonstrate that the adBNST Sst-neurons can be considered as a projection neuron population. To the best of my knowledge, this is the first study describing projection sites of the BNST Sst-neurons.

The projections were observed in brain regions known to control motivational states (NAc, VP), stress-related and defensive behaviours (MeA, CeA, LH, PAG), social behaviours (MeA, PAG), and autonomic and homeostatic regulation (LH, PBN). In much lesser extent, projections were also seen in the frontal cortex, and in the midbrain structures like the VTA, and *substantia nigra*. From the projections shown here, the BNST-PBN connection via Sst-neurons had been shown before by several studies, and can be therefore considered to be already confirmed (Moga et al. 1989; Magableh and Lundy

2014). The observed projection areas are all known to be output regions of the BNST, and reflect well the role of the structure in stress-control and valence monitoring (Lebow and Chen 2016). More precisely, the results of the present study suggest that the adBNST Sst-neurons could participate in modifying social behaviours and social stress through its projection to the CeA, MeA, and PAG, in modification of the reward system through the NAc, and VP, and in controlling homeostatic state – most likely in response to a presence of a stressor – through the LH, and PBN. In addition to these projections, fluorescent neurites of the adBNST Sst-neurons were observed widely in the BNST subnuclei, indicating at least some level of local innervation.

One limitation to this tracing study, and the main reason limiting the definitive concluding of the projection sites, is the one-way character of the tracing. The viral construct used in the study was an anterograde tracer, designed to move from the somatodendritic site towards the axon (Chamberlin et al. 1998). Since definitive markers of innervation site, like perisomatic baskets, were not observed, it is impossible to say in most of the cases, if the neurites observed are innervating the area or only moving through the region to innervate another (Dabrowska et al. 2016). This is especially true in the LH, through which several well-known nerve fibre bundles, like the medial forebrain bundle, are known to project (Chi and Flynn 1971; You et al. 2001). This question could be clarified by using sagittal and horizontal sections in addition to coronal slices; this could make it possible to see the endpoints of the neuronal bundles. Another way to address the problem would be with a combination of anterograde and retrograde tracers, infusing the here described projection sites, discovered through anterograde tracing, with the retrogradely moving tracer and seeing if the fluorescent marker can be seen together with Sst-marker in the BNST (Tomioka et al. 2005; Kim et al. 2013). This would also enable better subregion-level spatial resolution, as here the spread of the injected virus causes neurons from several BNST nuclei to be transfected. Because of this, it is not possible to differentiate the output regions between their projection origins in this study.

The above-mentioned double-labelling is another caveat in this study, because the co-labelling of Sst-tdTomato and GFP of the tracer was observed only in the injection site somas (Figure 7B), and in only a few neurites very near the BNST. The projection sites

postulated in this work are therefore based only on the qualitative assessment of the presence of the GFP-fluorescent neurites. Firstly, to more certainly demonstrate the co-localisation of the two fluorescent proteins, a quantitative method should be used (Pompey et al. 2013). The reason for the second problem, the lack of robust visible co-localisation, is not known, but tdTomato not being expressed in, or not being transported from the soma to the distal branches of the axon, was hypothesized. Even though there are known caveats in mouse reporter lines in general, there is no published evidence to back up the theory, which is also contradictory with the fact that rather robust neurite bundles are visibly expressing tdTomato in several brain regions (Figures 7E and 8A; Chen et al. 2015). Therefore, a problem of more technical nature is more likely, and this way, probably solvable by repeating the study.

A way of estimating the possibility of the Sst-innervation in the postulated projection areas is by looking at the known expressions of the Sst-receptors these regions, considering the prevalence of the receptor as an indicator of Sst-signalling at the site. Both the BNST and the amygdala are well known to have wide expression of several subtypes of Sstr: the potential projection sites in mind, both the CeA and MeA have been shown to express Sstr subtypes 1, 2, 3, and 4 (Table 1; Patel 1999; Stengel et al. 2012; Theodoropoulou and Stalla 2013) The wide expression of receptors is also true to the hypothalamus, PAG, and PBN, all expressing mostly $Sstr_1$ and $Sstr_3$, but not $Sstr_2$. From the observed sites with fluorescent fibres, only the VP and the NAc are known to be sparse in Sstr expression. Of course, the expression of Sstr has only instrumental value in estimating whether a brain area is an output region of the Sst-neurons. Still, the minor expression of Sstr in the NAc and VP, together with the observation made in this study showing that the presence of GFP-positive fibres in the anterior regions of the NAc and VP are sparse, and that the both brain regions are at least partly in the *ansa peduncularis*, it is possible that these two areas are not actual output regions for the adBNST Sst-neurons (Larriva-Sahd 2004; Larriva-Sahd 2006). Meanwhile the other postulated projection areas, including the BNTS nuclei, are known to express Sstr, and therefore are quite possibly true projection sites for the adBNST Sst-neurons. Further studies are still needed to confirm this.

7.2 Behavioural effects of the adBNST Sst-neuron activation

In order to elucidate the role of the adBNST Sst-neurons in the control of behaviour, I ran a set of behavioural tests combined with DREADD-mediated activation of the Sst-neurons. The tests were originally chosen, before the tracing study, on the basis of the best known BNST-mediated behaviours displayed by the earlier studies: the BNST is known to elicit robust changes in anxiety-like behaviours, and therefore the EPM and OF tests were included; the BNST-VTA projections have been shown to affect reward-associated behaviour, and this was tested by the CPP test; and noradrenergic signalling in the BNST is known to be important in the opioid withdrawal, which led into including the naltrexone-precipitated morphine withdrawal test (Aston-Jones et al. 1999b; Delfs et al. 2000; Jennings et al. 2013; Kim et al. 2013; Mazzone et al. 2016).

The anxiety tests, EPM and OF, failed to show any anxiogenic or anxiolytic effects following the DREADD-mediated activation of the adBNST Sst-neurons (Figure 9). Several studies have shown very robust changes in the anxiety-like behaviours after the activation of the BNST: for example, the DREADD-induced activation of GABA-neurons in the BNST was shown to significantly reduce the time the mice spend in the open arms in the EPM test (Mazzone et al. 2016). On the other hand, Kim et al (2013) showed that the optogenetic activation of the oval BNST neurons caused anxiogenic effects, while the activation of the anteromedial and the anterolateral BNST caused anxiolytic effects. This in mind, it should be noted that the spread of the DREADD-expression is wide enough to involve several BNST subnuclei: the most abundant Sst-expression is observed in the oval BNST, but Sst-neurons are also found in the anteromedial and anterolateral BNST (Figure 7A; Moga et al. 1989). Therefore, it is possible that the simultaneous activation of the Sst-neurons in the different subnuclei causes the activation of opposing neuronal pathways, leading to the situation with no behavioural phenotype. This problem could be tackled with optogenetics. Even if the DREADD-system has several strengths over the optogenetics, like that it does not depend on head-mounted devices possibly altering the animal behaviour on its own, optogenetics can potentially offer better spatial precision. This is due to the need to place the light-source – needed for the modulation of the genetically expressed ion channels – to the brain region of interest in addition to the receptor gene expression itself, whereas the

pharmacological modulation of the DREADD-system affects all the transfected cells despite the localisation (Deisseroth et al. 2006; Aravanis et al. 2007; Zhu and Roth 2015). Similar balancing-out effect could stem from the co-expression of Sst and CRF, which has been shown to occur in the oval BNST (de Miguel et al. submitted). Sst is commonly considered to suppress the CRF-mediated stress signalling, and if the DREADD-induced activation was enough to activate the both signalling pathways, their countering effects could again lead to the lack of anxiety phenotype in EPM and OF (Shibasaki et al. 1988; Stengel and Tache 2017).

Reflecting the results of the tracing studies done here, other kinds of anxiety-related endpoints could also be considered. The adBNST Sst-neurons were shown to send robust projections to the MeA, which is known to be a part of the social behaviour network (Figure 7D; Newman 1999, Goodson and Kabelik 2009). Because of this, anxiety tests incorporating social interaction or a predator simulation could show changes in the behaviour better than the approach-avoidance based tests used in this study (Calhoon and Tye 2015). Indeed, it has been shown that the exposure to predator ferret odour increases c-Fos activation of the Sst-neurons in the rat MeA (Butler et al. 2012). Contradicting the presented hypothesis, the same study showed increased c-Fos activation also in response to EPM test. Also, because the adBNST Sst-neurons were shown to send projections to homeostasis controlling brain regions, hypothalamus and PBN, changes in vital signs like the heart rate and breathing could be measured, as they are also known to be effected as a reaction to a stressor.

Similarly to the anxiety tests, the CPP test failed to show any rewarding or aversive effects in response to the DREADD-induced activation of the adBNST Sst-neurons (Figure 10). If we consider the lack of anxiety-related behavioural phenotypes to be caused by the intrinsic properties of the Sst-neurons and not by any technical problem, this finding could be seen to be in line with previous data showing that the activation of the BNST signalling pathways that cause increase in the anxiety levels, also cause place aversion, and that the anxiolytic pathways are linked with place preference (Kim et al. 2013; Mazzone et al. 2016). Since the adBNST Sst-neuron activation did not elicit changes in anxiety-related behaviours, it could be considered that the lack of observable effect on reward-related

behaviours is also presumable. The problem with this explanation is that the BNST- and the amygdala-associated reward behaviours are almost always somehow linked to the midbrain dopamine system, and the tracing study showed virtually no input from the adBNST Sst-neurons to the VTA or *substantia nigra*. Especially the lack of input to the VTA could be seen as an explanation for not observing rewarding or aversive behaviours in the CPP. The adBNST Sst-neurons were, on the other hand, shown to project to other reward-related brain areas like the NAc, VP, and PAG, which could affect the reward behaviour (Figure 7C). Yet, if the earlier prediction that the NAc and VP are not real projection sites for the Sst-neuron population is true, the lack of sufficient input to the reward-associated brain areas could explain also the absence of reward-related behavioural phenotypes in the CPP test.

Also, the naltrexone-precipitated morphine withdrawal test failed to show any changes in the measured withdrawal symptoms (Figure 11). Noradrenergic signalling from the medulla and *locus coeruleus* to the BNST has been implicated in opiate withdrawal, and noradrenaline is considered to activate the BNST CRF-signalling (Aston-Jones et al. 1999; Delfs et al. 2000; Silberman and Winder 2013). Even if the ventral BNST is most often linked with the opiate withdrawal, the oval BNST, subregion with most abundant expression of Sst-neurons, is known to have significant expression of adrenergic receptors, indicating noradrenergic innervation (Delfs et al. 2000; Bota et al. 2012). Since the Sst-signalling has been shown to counteract the effects of CRF, the activation of the adBNST Sst-neurons was hypothesised to attenuate the withdrawal symptoms (Shibasaki et al 1988; Stengel and Tache 2017). The lack of observable differences in naltrexone-precipitated morphine withdrawal-induced somatic symptoms could be explained by circuitry differences: two studies have shown that modulation of noradrenaline signalling in the BNST causes robust changes in withdrawal-related behaviours, intra-BNST β-adrenergic antagonist infusion attenuating the place aversion, but not the somatic withdrawal signs of morphine withdrawal (Aston-Jones et al. 1999; Delfs et al. 2000). This has led to the assumption that the BNST is relaying the affective component of the withdrawal symptoms, but not the somatic components, which would be in line with the results of this study.

The common characteristics of the neuropeptides that are secreted as signalling molecules, such as Sst, could in part explain the lack of observation in all the behavioural paradigms used in this test. As noted before, the release of Sst is slower than that of classical neurotransmitters, and its effect lasts longer (Baraban and Tallent 2004). Because of this, the role of the Sst-neurons could be more of a fine tuner than that of robust controller, like in the described example of the claustrum Sst-neurons, in which the Sst conveyed the changes in the action potential duration, while the inhibitory post-synaptic currents were mediated by GABA (Tang and Augustine 2015). This could mean that the activation of the adBNST Sst-neurons alone is not enough to produce robust behavioural changes, explaining the results of the behavioural test in this study. However, this explanation does not fully consider the known variety in the co-expression of Sst and other transmitters and neuropeptides. In many brain region, Sst is known to most often co-express GABA, and the modulation of the BNST GABAergic neurons has been shown to cause behavioural changes: Mazzone et al. (2016) were able to show robust decreases in open-arm entries in EPM test and in the time spent in light in the light-dark box test in response to hM3Dq-DREADD activation of the BNST GABA neurons by using vesicular GABA transporter VGAT-cre mice. This does not necessarily contradict the idea of the fine-tuning nature, as the adBNST Sst-neurons could be too small a population of neurons that the effect of their classic transmitter release might not be enough to cause changes in behavioural phenotype. The tests were done with healthy mice with no preliminary treatments of challenges, which could also affect the results, as it is known that not all the inhibitory or activating methods cause behavioural changes in otherwise untreated animals; for example, the DREADD-mediated inhibition of the BNST GABA-neurons alone was shown not to cause any observable anxiety-related behavioural changes (Mazzone et al. 2016). Preliminary stressor, like forced swimming or movement restriction, before the anxiety tests could cause symptoms that the activation of the adBNST Sst-neurons could alleviate. Also, inhibition of the neuron population, instead of activation, could yield different results.

Related to the transmitter arguments, it should be noted that no wider histological characterisation of the adBNST Sst-neurons was done, and therefore no knowledge of their neurotransmitter profile is available. Also, there is no certainty that the DREADD-

induced activation of the neurons is causing release of Sst. The DREADDs are inherently G-protein coupled receptors which cause more long-lasting effects through a variety of intracellular signalling pathways, as opposed to fast and rather robust effects of the ion channels (Armbruster et al. 2007; Alexander et al. 2009). Because the neuropeptides are known to need repeated, high frequency stimulation in order to be released, the G-protein coupled activation might not be enough. This question could be addressed with, for example, *ex vivo* electrophysiological measurements. Also, with the presented data it is not clear if the DREADDs activated the neurons at all. The lack of intended Sst-neuron activation would certainly explain the absence of the behavioural phenotypes. The functionality of the DREADD-system (both, the used vectors, and the activating ligand) could be tested with electrophysiology, or by finding out if the CNO administration causes changes in c-Fos expression in the transfected area compared to the sham control.

DREADDs carry some methodological caveats, in addition to the ones already touched upon, that should be also considered when interpreting the behavioural data. As mentioned, DREADDs work through the G-protein coupled systems (Armbruster et al. 2007; Alexander et al. 2009). Even if the expression of the designer receptor itself is a result of the use of genetic tools, the intracellular signalling molecules are of endogenous origin and the activation of the DREADDs might in the end have other, even longer lasting effects than the modulation of the neurons firing rate. Possibly relating to this, some groups utilising this chemogenetic technique have reported observable *in vivo* compensatory effects to take place as a response to the DREADD-induced modulations (Klapoetke et al. 2014; Saloman et al. 2016). For example, Saloman and colleagues discovered that the expression of inhibitory hM_4D_i-DREADD receptors in sensory neurons caused changes in Ca^{2+} and Na^{+} currents even in the absence of the ligand CNO. Also, very recently, some discrepancy concerning the role and the nature of the most widely used DREADD-ligand, CNO, has surfaced: Gomez et al. (2017) claimed that it is not CNO, but clozapine, product of in-system conversion of CNO, that causes the activation of the DREADDs. While conversion of CNO back into clozapine has not been shown to happen in mice, the conversion would have implications not only to the method itself, but also more acutely to this study because clozapine has been shown to have direct effects on Sst-expression (Salin et al. 1990; Arif et al. 2006).

10 CONCLUSIONS

This study revealed that the Sst-neurons in the adBNST send projections outside the BNST nuclei, to several brain regions known to control fear and anxiety behaviours, as well as stress responses, and homeostatic balance. While the nature of the neuronal tracing method used in this study requires further research to confirm the observations made here, the postulated projection areas are well in line with the earlier research conducted on the connectivity of the BNST, and reflect the known functional and behavioural roles of the region.

The behavioural research conducted in this study failed to reveal any significant behavioural effects for the activation of the adBNST Sst-neurons. These tests were the first ones performed to characterize the functional properties and behavioural role of the adBNST Sst-neurons, conducted in healthy animals, with no prior challenge or treatment, and are therefore not to be interpreted as a failure. While some methodological caveats could contribute to the result, and could be addressed with more accurate and site-specific technologies, in a wider perspective more thorough characterization of the adBNST Sst-neuron population is needed to uncover its functions in the healthy brain. This is required to seek novel strategies to modulate this one – or any – neuron subpopulation, and to consider it as a future drug target in anxiety- and addiction related disorders.

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